Application
for
United States Letters Patent

To all whom it may concern:

Be it known that we,

TIKVA VOGEL, AVIGDOR LEVANON, MOSHE WERBER, RACHEL GUY AND AMOS PANET

have invented certain new and useful improvements in

FIBRIN BINDING DOMAIN POLYPEPTIDES AND USES AND METHODS OF PRODUCING SAME

of which the following is a full, clear and exact description.

Dkt. 25775-C JPW/SJT

FIBRIN BINDING DOMAIN POLYPEPTIDES AND USES AND METHODS OF PRODUCING SAME

INS a1

a1 ~~This application is a continuation-in-part of U.S.~~ Serial No. 345,952, filed April ~~28, 1989~~, which is a continuation-in-part of U.S. Serial No. 291,951, filed December ~~29~~, 1988, the contents of both of which are ~~hereby incorporated by reference into this application.~~

Background of the Invention

Throughout this application, various publications are referenced by Arabic numerals within parentheses. Full citations for these references may be found at the end of the specification immediately preceding the claims. The disclosures of these publications in their entireties are hereby incorpcrated by reference in this application in order to more fully describe the state of the art to which this invention pertains.

Atherosclerosis is the progressive narrowing of the lumen (inner passageway) of arterial blood vessels by layers of plaque (fatty and fibrous tissues). Atherosclerosis can occur in any artery. In coronary arteries it may result in heart attacks; in cerebral arteries it may result in strokes; and in peripheral arteries it may result in gangrene of the legs and feet.

Atherosclerosis is the single largest medical problem currently facing the United States and other developed countries. Approximately 40 million people in the United States are at risk of developing ather sclerosis. However, only 6 million pe ple in the United States sh w overt signs of the disease. The rest remain undiagnosed until the disease manifests

itself symptomatically, in the worst case as heart attack or stroke. Heart attack and stroke, respectively, represent the first and third leading causes of death in the United States. Over 500,000 people die of heart attacks every year and a significant sub-group of these patients expire without warning.

The endothelium is located between the blood and arterial tissue and serves as a barrier against the accumulation of blood components in the vascular wall. Formation of atherosclerotic lesions (plaques) in the sub-endothelium is associated with major coronary artery disease and stroke. The causes and detection of such lesions have been intensely investigated.

Endothelial injury is believed to be an initial step in the formation of the atherosclerotic lesions and may be caused by, i.e., hemodynamic strain, hypercholesterolemia, hypertension and immune complex disease. Endothelial injury leads to thickening of the intima, cell proliferation, cholesterol accumulation, and formation of connective tissue fibers. IgG and complement factor C3 accumulation in injured endothelial cells and nonendothelialized intima has been observed. Mononuclear cells derived from blood are also part of the cell population in atherosclerotic lesions. The mechanism of plaque formation is not fully known. However, a probable mechanism is that the earliest lesions, fatty streaks, consisting of mixtures of T cells and monocyte-derived macrophages, form in the subendothelium followed by a secretion of various cyt kines, which leads to a migration of smooth cells into the intima and their accumulation.

Alth ugh atherosclerosis is generally a diffuse disease, human coronary atherosclerosis lends itself to bypass procedures because the major site of plaque formation is usually proximally distributed. As a result, direct coronary artery bypass has become the most frequently selected form of myocardial revascularization. The aorta-coronary artery vein graft of the internal mammary artery graft have become technically standardized and have high long-term patency rates. These long-term results, however, can be compromised by progressive atherosclerotic lesion distal to the graft anastomosis. Other cases are inoperable because of distal disease. Previously, distal lesions have been ignored or, in selected cases, treated by endarterectomy although neither approach has proved entirely satisfactory.

Most existing procedures for the diagnosis and treatment of atherosclerosis are invasive, costly, and of limited effectiveness in a significant percentage of patient cases.

The concept of plaque enhancement by application of a stain has been reported [Spears, J. et al., J. Clin. Invest. 71: 395-399 (1983)]. These stains mark the plaque surfaces with a fluorescent compound. Plaque destruction by photoactivation of hematoporphyrin derivatives using an intraluminal laser-transmitting optical fiber has been suggested [Abela, G. et al., Am. J. Cardio. 50: 1199-1205 (1982)]. Moreover, tetracycline stains have also been suggested. [Murphy-Chutorian, D. et al., Am. J. Cardiol. 55: 1293-1297 (1985)].

The above-identified stains were selected for their ability to bind to components of the atherosclerotic plaque. In principal, the stain absorbs laser light concentrating the light at the stained surface. Some staining of healthy tissue occurs causing stain associated damage to the surrounding tissue. Because laser light wavelength is limited to the absorption wavelength of the stain, chromophores offering optimum absorption of laser light must be used to provide best controlled ablation.

Imaging and detection of coronary thrombi, pulmonary emboli, deep venous thrombosis and atherosclerotic lesions are of great clinical importance especially in view of the new thrombolytic agents which have recently been developed. Several experimental approaches for non-invasive detection of thrombi by use of radiopharmaceutical agents have been reported but none has gained wide clinical recognition because of intrinsic drawbacks associated with each agent.

The basic characteristics of a radiopharmaceutical for early detection of intravascular atherosclerotic lesions and thrombi are the following: (i) high affinity for thrombus components; (ii) relatively fast pharmacokinetic blood clearance rate [in order to obtain a high ratio of thrombus (bound) to blood (unbound) radiolabeled tracer]; (iii) safety: non-toxic and non-immunogenic; and (iv) simplicity of preparation and use.

The various agents for imaging thrombi described in the literature and their drawbacks are as follows: (a) aut logous platelets labeled with ^{111}In: the procedur is cumbersome, time consuming and the blood clearance

tim is relatively long, viz. 2 days (2); (b) ^{131}I-fibrinogen: the assay is based on the (low) affinity of injected radiolabeled fibrinogen for the thrombus but it is not suitable for rapid imaging tests because of its long residence time in blood and furthermore it does not become incorporated into older thrombi nor is it incorporated in the presence of heparin (3, 36); (c) fragment E1 of human fibrin: although it seems superior to fibrinogen it is difficult to prepare in sufficient quantities for widespread clinical use (4); (d) mouse anti-fibrin monoclonal antibodies: although they are specific and have high affinities to thrombi they have a relatively long blood clearance time and are potentially immunogenic to human subjects (5, 33, 34); (e) mouse monoclonal antibodies specific for activated platelets (6, 7): disadvantage as (d); and (f) labeled fibronectin (1): although fibronectin (see below) has an affinity for a number of substances occurring in thrombi it has a relatively long blood clearance time and the buildup of radioactivity in the thrombus is slow. Thus there is a need in the art for a thrombus-specific radiopharmaceutical for rapid imaging of thrombi.

U.S. Patent 4,343,734 (Lian et al.) describes specific gamma-carboxyglutamic acid (GLA) antibodies which can be labeled with fluorescein for immunofluorescence staining of tissue to determine the presence therein of GLA. Specific GLA antibodies bind to GLA which is present in advanced atherosclerotic plaque, having calcium deposits. Lian et al. report that GLA is not found in uncalcified plaques and that GLA is found in cardiac valves and a rtas, and in circulating proteins such as prothrombin, clotting factors VII, IX and X, Protein C and Protein S. How ver, th GLA binding

antibodi s of Lian et al. do not selectively bind to atherosclerotic plaque.

Fibronectin is a glycoprotein composed of two identical subunits each of approximately 220,000 molecular weight. Two major forms of fibronectin are produced and secreted by human cells in culture and in vivo (8). The cell-associated fibronectin is relatively insoluble and participates in cell adhesion, wound healing, cell differentiation and phagocytosis. The plasma fibronectin, produced primarily in the liver, is a soluble serum protein with biological properties similar to those of cell fibronectin.

Fibronectin is considered a multifunctional modular protein since limited proteolytic cleavage produces polypeptides with distinct activities. The different functional domains of the fibronectin molecule have been obtained and defined by partial proteolytic digestion, and include heparin, DNA, fibrin, gelatin, and cell binding domains (8-13).

Baralle, F.E., European Patent Publication No. 207,751, published January 7, 1989, discloses the complete cDNA sequence of fibronectin. Baralle also discloses the expression of fusion proteins containing a portion of the collagen binding domain of fibronectin fused to the Escherichia coli protein ß-galactosidase. Similar fusion proteins are disclosed by Owens and Baralle (14). Obara et al. (1987) disclose the expression of a portion of the cell binding domain of human fibronectin fused to Escherichia coli ß-galactosidase (15). Additionally, Obara et al. (1988) disclose the expression of portions of th c ll binding domain fused to ß-galactosidase which have been mutag niz d, i.e., sit

specific deletions of portions of th cell binding domain were obtained as fused proteins (16). The carboxy terminal fibrin-binding domain of human fibronectin has been expressed in mouse L cells as a fusion protein with the signal sequence of human protein C inhibitor (17).

None of the above references discloses the expression of the N-terminal fibrin binding domain of fibronectin; furthermore all the recombinant proteins they disclose are expression of fusion proteins.

This invention provides polypeptides having an amino acid sequence substantially present in the N-terminal fibrin binding domain of fibronectin. These polypeptides have varying molecular weights (31 kD, 20 kD and 12 kD), as defined by comparison markers on SDS gels under reducing conditions, and have the following characteristics which make them promising pharmaceutical agents: (i) have an amino acid sequence present in a human protein and thus are contemplated to not be immunogenic; (ii) high affinity to fibrin and able to become covalently cross-linked to growing as well as to preformed thrombi (clots); (iii) bind to extracellular matrix, which property may be exploited to detect atherosclerotic plaques; (iv) have a relatively short blood clearance time; (v) incorporate into clots in the presence of heparin; and (vi) are produced by recombinant techniques and can therefore potentially be manufactured on a large scale.

The subject invention provides an inexpensive, accurate method for imaging fibrin-containing substances, i.e., a thrombus and atherosclerotic plaque, both *in vitro* and *in vivo*. In addition, the subject inventi n

provides plasmids for expressing polypeptides having an amino acid sequence substantially present in the fibrin binding domain of naturally-occurring human fibronectin and capable of binding to fibrin which are labeled and used for imaging the fibrin-containing substances, and methods of producing such polypeptides.

These polypeptides may also be used as anti-infective agents. The involvement of fibronectin in adhesion to, and invasion of, wounds by a wide range of gram positive bacteria is well established (18, 19). The polypeptides of the fibrin binding domain of fibronectin according to this invention may be used as anti-infective agents to prevent sepsis in wounds.

Summary of the Invention

This invention provides an imaging agent which comprises a polypeptide labeled with an imageable marker, such polypeptide having an amino acid sequence substantially present in the fibrin binding domain of naturally-occurring human fibronectin and being capable of binding to fibrin.

Further provided is a method for imaging a fibrin-containing substance, i.e. a thrombus or atherosclerotic plaque, which comprises contacting the fibrin-containing substance to be imaged with the imaging agent disclosed above under conditions such that the agent binds to the fibrin-containing substance and imaging the bound agent and thereby imaging the fibrin-containing substance.

Also provided is a plasmid for expression of a polypeptide which having an amino acid sequence substantially present in the fibrin binding domain of naturally-occurring human fibronectin and being capable of binding to fibrin comprising DNA encoding the polypeptide and DNA encoding suitable regulatory elements positioned relative to the DNA encoding the polypeptide so as to effect expression of the polypeptide in a suitable host cell.

The invention also provides a purified polypeptide substantially free of other substances of human origin which has an amino acid sequence substantially present in the fibrin binding domain of naturally-occurring human fibronectin and being capable of binding to fibrin.

Further provided are methods of treatment using such polypeptides and methods of recovering and refolding and reoxidizing such polypeptides.

Brief D scription of the Figures

In the figures, the numbers in brackets adjacent certain of the restriction enzyme sites shown correspond to the identically numbered positions along the nucleotide sequence of human fibronectin cDNA as shown in Figure 1 (see also Figure 3 of Baralle, F.E., European Patent Publication No. 207,751, published January 7, 1987).

The following figures describe the construction of plasmids expressing polypeptides having an amino acid sequence substantially present in the amino-terminal fibrin binding domain (FBD) of fibronectin. The FBD commences at amino acid number 1 of mature fibronectin, which is glutamine and corresponds to the fourth amino acid ~~(G)~~ (Q) shown in Figure 1A, i.e., the N-terminus of the FBD sequence is ~~G-A-G-G~~ QAQQ (glutamine-alanine-glutamine-glutamine); the corresponding first nucleotide in the cDNA sequence of Figure 1A is therefore number 14, indicated by an arrow. All the recombinant FBD polypeptides described in these figures and throughout the specification are numbered from this first glutamine as amino acid number 1 and all the corresponding cDNA sequences are numbered as shown in Figure 1.

Some of the figures describe the construction of plasmids expressing an FBD polypeptide joined at its C-terminus to part of the cell binding domain (CBD) of fibronectin. The cDNA sequence corresponding to the CBD which applicants have cloned and expressed is missing the 270 bp extra d main (ED) s gment which ext nds from nucleotides 4812 t 5081, inclusive, on the Baralle map (see Figure 1). Thus, the cDNA

sequence which is said to extend from nucleotide 3317 to 5566 on the Baralle map, contains only 1980 nucleotides, because it is missing the 270 nucleotides of the ED segment, namely from nucleotides 4812 to 5081 inclusive; this region is also known in the art as the ED-A region; concomitantly amino acid 1690 is changed from alanine to threonine. Similarly, the polypeptide expressed by that DNA fragment would encode from amino acid 1102 to amino acid 1851 on the Baralle map but would be missing the 90 amino acids encoded by the ED region, namely amino acids 1600-1689 inclusive, and thus it would contain only 660 amino acids. This is true for all CBD polypeptides described in this application which span the ED region. (The region known in the art as the ED-B region is missing both in Baralle's sequence and in applicants' DNA.)

The definition of the polypeptides expressed as 31 kD, 20 kD, 12 kD and 33 kD is an operational definition, based on their mobility on SDS polyacrylamide gels under reducing conditions compared to that of markers of known molecular weight.

Figure 1. All panels (A-H) of this figure show ~~This figure shows~~ the nucleotide sequence (continued from panel to panel) of human fibronectin cDNA.

Figure 2. Seven pairs of chemically synthesized oligomers were prepared. The synthetic oligomers code for the first 153 N-terminal amino acids of human fibronectin (FN). This figure shows the sequence of these 7 pairs of synthetic oligomers.

Figure 3. The DNA fragment coding for amino acids 1 to 153 of th N-terminal domain of human FN was

assembled from the 7 pairs of chemically synthesized oligomers shown in Figure 3 as follows:

Oligomers 3/4, 5/6, 7/8 and 9/10, each pair in a separate tube, were annealed and then phosphorylated at the 5' end using T4 polynucleotide kinase enzyme.

In the second step, pairs 3/4 and 5/6 were ligated to each other using T4 DNA ligase. Similarly, reaction pairs 7/8 and 9/10 were ligated to each other. After each step of ligation an aliquot of the ligation mixture was analyzed on gel to determine the size of the newly formed fragments and the efficiency of ligation.

In the third step, the two above mentioned ligation mixtures were mixed together and pair 6, oligomers 11/12 which had been annealed and phosphorylated previously in a separate tube were added to the mixture. A 326 base pair DNA fragment obtained from the above ligation mixture was isolated from an agarose gel and purified.

The purified synthetic 326 fragment was added to two additional pairs of synthetic linkers: Pair 1, oligomers 1/2 and Pair 7 oligomers 13/14. In Pair 1 only oligomer 2 was phosphorylated at the 5' end and in Pair 7 only oligomer 13 was phosphorylated at the 5' end.

After ligation with T4 DNA ligase the mixture without any further isolation was added to pBR322 vector DNA digested with EcoRI and BamHI endonucleases.

The plasmid obtained, designated pFN 932-18 contained the entire synthetic EcoRI (5' end) - BamHI (3' end) restriction fragment coding for the N-terminal 153 amino acids of human FN, in a pBR322 vector.

Figure 4. Expression of the N-terminal 153 amino acid sequence of FN.

Plasmid pFN 932-18 was digested with NdeI and BamHI endonucleases. The NdeI-BamHI DNA fragment coding for FN (first 153 amino acids + additional N-terminal methionine) was isolated and ligated into the large fragment obtained by digestion of plasmid pTV301 with NdeI and BglII endonucleases. (Plasmid pTV301 (Figure 33) expresses human growth hormone, hGH, under the control of λ P_L promoter and the cII RBS).

The plasmid obtained was designated pFN949-2.

Figure 5. Insertion of termination codon TAA at the 3' end of the N-terminal domain of FN (at amino acid 262)

A synthetic oligonucleotide containing a TAA termination codon and a BglII site having the following sequence:

CTGTTTAAGCA
GACAAATTCGTCTAG

was ligated to the 3' end (PvuII site) of an EcoRI-PvuII FN fragment isolated from cDNA clone plasmid p931-5 (see Figure 5) digested with EcoRI and PvuII. The ligation was carried out in the presence of DNA vector plasmid pBR322 digested with EcoRI and BamHI

(large fragment). The plasmid obtained was designated pFN935-12.

Figure 6. Subcloning of the carboxy-terminal region of FBD in λ P_L expression vector

Plasmid pFN 935-12 was digested with EcoRI and HincII. The EcoRI-HincII fragment coding for FN was isolated and ligated to DNA, the large fragment obtained by digestion of plasmid pTV194-80 with EcoRI and SmaI. (Plasmid pTV194-80 expresses human ApoE under the control of the λ P_L promoter and ß-lactamase promoter and RBS). The plasmid obtained was designated pFN 946-12. This plasmid is deleted of the PBLA sequences and therefore does not express the carboxy domain of FBD.

The construction of pTV194-80 from plasmid p579 (Figure 34) is shown in Figures 30 and 31.

Figure 7. Construction of the DNA fragment coding for the FBD from nucleotide No. 14 to nucleotide No. 599

Three pairs of chemically synthesized oligomers with the following DNA sequences:

Pair 1

#15 5'-TGAGAAGTGTTTTGATCATGCTGCTGGGACTTCCTATGTGG-3'

#16 3'- CTTCACAAAACTAGTACGACGACCCTGAAGGATACACCAGCCT-5'

Pair 2

#17 5-TCGGAGAAACGTGGGAGAAGCCCTACCAAGGCTGGATGATGGTAG-3'

#18 3- CTTTGCACCCTCTTCGGGATGGTTCCGACCTACTACCATCTAACA-5'

Pair 3

#19 5'-ATTGTACTTGCCTGGGAGAAGGCAGCGGACGCATCACTTGCACTT-3'

#20 3'- TGAACGGACCCTCTTCCGTCGCCTGCGTAGTGAACGTGAAGATC-5'

were used to carry out this construction.

Oligomers 15/16 and 17/18 were annealed and phosphorylated at the 5' end each in a separate tube and then mixed together for ligation using T4 DNA ligase. After 3 hours of ligation, oligomers 19/20 (previously annealed and kinased at their 5' ends) were added for an additional 3 hours ligation at room temperature.

The synthetic DNA fragment obtained was used for further ligation with an EcoRI-DdeI FN coding sequence obtained from plasmid pFN 932-18 digested with EcoRI and DdeI. The ligation was carried out in the presence of plasmid pUC19 digested with EcoRI and XbaI (large fragment).

The plasmid obtained was designated pFN948-4.

Figure 8. Construction of the entire FBD region

Plasmid pFN948-4 was digested with EcoRI and XbaI. The EcoRI-XbaI fragment coding for the N-terminal region of FBD was isolated and ligated to the carboxy terminal region of FBD by digestion of plasmid pFN946-12 with EcoRI and XbaI (using the large fragment). The plasmid obtained was designated pFN 957.

Figure 9. Expression of the entire FBD polypeptide under the control of the λ P_L promoter and λ cII RBS

Plasmid pFN 957 was digested with NdeI and HindIII. The NdeI-HindIII fragment coding for the FBD was isolated and ligated into the isolated vector fragment of plasmid pTV301 digested with NdeI and HindIII in the presence of isolated purified HindIII-HindIII T_1T_2 coding DNA fragment. The plasmid obtained was designated pFN962-3.

Figure 10. Expression of the entire FBD polypeptide under λ P_L promoter and PBLA ribosomal binding site

Plasmid pBLA11 (ATCC Accession No. 39788) was digested with EcoRI and AluI. The EcoRI-AluI fragment coding for the ß-lactamase promoter and ß-lactamase RBS was isolated and ligated into plasmid pFN962-3 (Figure 9) digested with NdeI, then treated with Klenow enzyme in the presence of all four dNTPs to fill in the NdeI site and digested with EcoRI (using the large fragment). The plasmid obtained was designated pFN 975-25.

Figure 11. Refolding/reoxidation and purification of r31 kD as followed by SDS-PAGE under reducing and non-reducing conditions

The gel (12% acrylamide) under reducing conditions (with ß-mercaptoethanol (ME)) monitors the process of purification, whereas the non-reducing conditions (without ME) are indicative of the refolding/r oxidation, l ading to fast r moving and less diffuse bands. Note (in the absence of ME) that the band of 'Aft r Phenyl-S' is much

sharper than that of 'Refolded', indicating that reoxidation continues even during the purification.

Refolded (GSH/GSSG):r31kD which has been refolded/reoxidized - after having been extracted from the crude pellet in the presence of GSH/GSSG 3 mM/0.3 mM at pH 8.0; Phenyl-S; Phenyl-Sepharose; Q-S; Q-Sepharose; p31 kD; plasma-derived 31 kD (obtained by tryptic digestion); molecular weight markers: Low Molecular Weight protein calibration kit (Pharmacia Fine Chemicals), containing markers whose molecular weights are 94 kD, 67 kD, 43 kD, 30 kD, 20.1 kD and 14.4 kD.

Figure 12. Purification of GSH/GSSG-refolded r31 kD by Phenyl-Sepharose chromatography

A suspension of refolded/reoxidized and "scrambled" 31 kD, as well as insoluble contaminants, which has been extracted from 10 grams of pellet, was subjected to centrifugation at 13,000 rpm (see Section 3.1 in Example 5). The supernatant (1,280 ml) was brought to 0.2 M in ammonium sulfate (AS) and loaded onto a 45 ml column of phenyl-Sepharose previously equilibrated with Buffer A at pH 8.5, containing also 0.2 M AS. The column was washed with 150 ml of the same solution, followed by 150 ml of Buffer A, 50 ml of water and 50 ml of 6 M GuCl. The purified r31kD appeared in the Buffer A fraction and at this stage it was more than 85% pure. Absorbance was measured at 280 nm.

Figure 13. Concentration and purification of r31 kD by Heparin-Sepharose chromatography

Approximately 1/2 of the Buffer A peak from the phenyl-Sepharose step (see Figure 12) was conc ntrated and

purified on a 10 ml Heparin-Sepharose column, from which it was eluted by a solution of 0.5 M NaCl in Buffer A. At this stage the r31 kd is more than 90% pure. Absorbance was measured at 280 nm.

Figure 14. Purification of r31 kD by Q-Sepharose chromatography

Concentrated r31 kD (Figure 13), which had been dialyzed against Buffer A, pH 8.5, was loaded on a 40 ml column of Q-Sepharose, which had previously been equilibrated with the same buffer. The purified r31 kD, which eluted in the flow-through and wash fractions, was concentrated by lyophilization. The column was washed free from the contaminant proteins by a step of 1 M NaCl. The purified r31 kD is at this stage more than 95% pure. Absorbance was measured at 280 nm.

Figure 15. Analytical FPLC-gel permeation of r31 kD on a Superose 12 column

A mixture of plasma derived and recombinant 31kD (100 µl at 0.8 mg/ml) in running buffer (20 mM Tris.HCl - 150 mM NaCl. pH 7.8) was applied onto the Superose 12 column (HR 10/30), pre-equilibrated in running buffer, and eluted from it in the same buffer. Flow rate - 0.4 ml/minute; chart speed - 0.25 cm/minute; absorption units full scale - 0.1; detection wavelength - 280 nm; run time - 60 minutes. The retention times of both the plasma derived and recombinant 31kD, when run separately, was identical to that obtained for the mixture, and correspond to an apparent molecular weight of 37 kD.

Figure 16. Comparison of the pharmacokinetics of fibronectin (FN) and r31 kD FBD in rats

^{125}I-FN (0.1 mg/kg; 5 x 10^6 cpm) or ^{125}I-r31 FBD (0.1 mg/kg; 5 x 10^6 cpm) were injected intravenously and at the time indicated blood samples were withdrawn. Insoluble radioactivity in the blood samples was determined by trichloroacetic acid precipitation; at zero time, the 100% value represents 40,000 cpm/ml for FN and 46,000 cpm/ml for FBD.

Figure 17. Binding of ^{125}I-r31 kD to fibrin: effect of thrombin and Ca^{++} ions

Reaction I was carried out as described in Example 7 using 20 µl citrated whole blood, 0.15 µM ^{125}I-r-FBD (r31 kD) (5.6 x 10^5 cpm/µg).

Reaction II was initiated with the formation of unlabeled Fibrin clot using 20 µl citrated whole blood as described in Example 7. After the first incubation, 0.15 µM ^{125}I-r-FBD was added to the existing reaction tube ("Serum") or to the Fibrin pellet following centrifugation and resuspension in PBS ("PBS"). When $CaCl_2$ and Hirudin were added the concentrations were 5 mM and 3 U/ml, respectively. Reaction II was continued thereafter as described in Example 7.

Figure 18. Release of ^{125}I-FBD (p31 kD) from Fibrin clot by plasmin

Reacti n I was carried out using several tubes containing 100 µl citrated whole blood and 0.3 µM ^{125}I-P-FBD (5.0 x 10^4 cpm/µg). At the end of the incubati n, the pell t

was collect d by centrifugati n and then resuspended in PBS solution containing 1 μ/ml plasmin (from porcine blood, Sigma) and was further incubated at 37°C for the indicated time intervals. The reaction was terminated by cooling and immediate centrifugation. The radioactivity in the supernatant and the pellet was measured by a gamma counter.

The pellet and the supernatant were resuspended in gel electrophoresis sample buffer (final concentration of 3% glycerol, 2% BME, 1% SDS, 0.2% Bromophenol Blue), boiled for 15 minutes, and electrophoresed in 10% PAGE-SDS. The gel was then autoradiographed on x-ray film. No radioactivity was detected from the pellet. Radioactivity from the supernatant was detected in a position corresponding to control ^{125}I-FBD incubated with plasmin (results not shown).

Figure 19.

The binding of 0.15 μM ^{125}I-r-FBD (^{125}I-r31 kD; 5.6 x 10^5 cpm/μg) to 20 μl citrated whole blood was performed using reaction I conditions (see Example 7). The reaction was performed either without competitors (C) or in the presence of various concentrations of unlabeled "folded proteins", "reduced" FBD and related molecules.

rI = "Fully reduced" r31 kD FBD
rII = "Reduced carboxyamidated" r31 kD FBD (i.e. reduced/blocked 31 kD FBD)
p = 31 kD FBD from Trypsin cleavage of plasma derived Fibronectin.

Figure 20. Binding of ^{125}I-r31 kD to fibrin (Reaction II). Effect of transglutaminase inhibitors and related molecules

Unlabeled fibrin clot was formed using 20 µl citrated whole blood and using the conditions described for reaction II in Example 7.

At the end of the first incubation period, and prior to the addition of r-^{125}I-FBD (0.15 µM; 2.9 x 10^4 cpm/µg), the indicated concentrations of spermidin, putrescine and FBD-"R" ("Reduced-carboxamidated" plasma derived FBD) were added to the specified reactions.

Figure 21. Binding of ^{125}I-r31 kD to preformed fibrin clot (Reaction II): effect of fibrin clot age

The unlabeled fibrin clot was formed using 20 µl citrated whole blood and using the conditions described for reaction II in Example 7.

At the end of the first incubation period, half of the samples were centrifuged and the fibrin pellet was resuspended in PBS solution containing 5 mM $CaCl_2$ (Reaction "C") or PBS containing 5 mM $CaCl_2$ and 300 µg/ml FN (Reaction "D"). No additions or changes were performed to the samples designated "A". 300 µg/ml FN was added to the sample designated "B". Subsequently, the reaction mixtures were allowed to incubate at 37°C for 1 hour, 4 hours, or 24 hours (as indicated in the figure). Then ^{125}I-r31 kD (0.15 µM final concentration, 5.4 x 10^5 cpm/µg) was added and th incubation was continued f r an additi nal 30 minutes. Th r action was terminated as described in Example 7.

Figure 22. Binding of ^{125}I-r31 kD to "Naive" Thrombi (reactions I and II): effect of thrombii age on the binding

Aliquots of 20 µl non-citrated fresh whole human blood were incubated in non-siliconized tubes at 37°C with either 0.15 µM ^{125}I-r31 kD or alone (reaction I and II, respectively). At the indicated time intervals, reactions were either terminated (reaction I) or 0.15 µM ^{125}I-r31 kD (2.9 x 10^4 cpm/µg) was added, and incubation terminated after an additional 2 hours (reaction II).

Figure 23. Binding of ^{125}I-r31 kD to Fibrin (Reaction I): Effect of Exogenous Transglutaminase and "Reduced" FBD

20 µl aliquots of noncitrated whole human blood were incubated with 0.15 µM ^{125}I-r31 kD (2.9 x 10^4 cpm/µg) alone ("control") or together with pig liver Transglutaminase ("control + T.G.", 0.2 units/ml; Sigma).

Some of the tubes contained "Reduced" (carboxamidated) p31 kD as indicated in the figure.

The addition of exogenous Transglutaminase to the binding reaction increased the binding values by more than a factor of two. When "reduced-carboxamidated" r31 kD was added to the reaction we observed a similar extent of inhibitory effect as with the exogenous factor XIIIa (inhibition of 53% and 71% by 0.3 µM and 3.0 µM, respectively), indicating an identical inhibitory effect of the reduced FBD on both types of Transglutaminase.

Figure 24. Binding of FBD to ECM

The biological activity of the FBD was studied in a model of vascular injury using the Extra Cellular Matrix, "ECM", of cultured endothelial cells (20).

33 mm ECM plates following 3x washing in PBS were incubated at 37°C in a CO_2 incubator with 0.5 ml DMEM-10% FCS containing 2.5 mM $CaCl_2$, 1 μ/ml Thrombin, and the indicated concentrations of ^{125}I-P-FBD (about 4.4 x 10^5 cpm/μg). Parallel plates were incubated in the absence of thrombin as indicated in the figure. Following 45 minutes of incubation, the plates were washed 3 times with 1 ml PBS, extracted with 0.5 ml of 0.1% SDS-PBS solution, and radioactivity was measured by a gamma counter. The values described in the figure represent an average of two plates.

Figure 25. Binding of FN and FBD to S. aureus

The binding reaction was carried out in a solution using 5 x 10^8 PFU/ml of S. aureus SA113 (ATCC Accession No. 35556) and ^{125}I-FN (4 x 10^4 cpm/μg) or ^{125}I-FBD (1.3 x 10^5 cpm/μg; r31 kD FBD, "reoxidized-refolded") at concentrations indicated in the figure and as described in methods. The concentration of the labeled molecules described is calculated using molecular weights of 220,000 and 31,000 daltons for FN and r31 kD, respectively.

Figure 26. Binding of FBD to S. aureus: competition with folded and reduced forms

Binding in solution of 1.25 μg ^{125}I-p31 kD (2.3 x 10^5 cpm/μg) to 5 x 10^8 PFU/ml of S. aureus SA113 was carried out in the presence of the indicated concentrations of the following unlabeled proteins: P-FBD (p31 kD), r-FBD (r31 kD FBD "reoxidized-refolded"), r-FBD-R (r31kD FBD "Reduced Carboxyamidated), and r-CBD (r-33 kD cell binding domain of FN). The binding reaction was carried out as indicated in the methods section.

Figure 27. Binding of S. aureus to immobilized FN

Binding of 7.5 x 10^8 PFU/ml of 3H-leucine-S. aureus (5.8 cpm/10^5 PFU) to FN immobilized onto plastic vials was carried out as described in methods and in the presence of human plasma FN, r-FBD (r31 kD FBD "reoxidized-refolded"), P-FBD (p31 kD), or BSA (Bovine Serum Albumin, Sigma). Binding of "control" reaction in the absence of competitors (9.3% of input bacteria) was normalized to 100%.

Figure 28. Binding of S. aureus to Catheters

Binding of 3.0 x 10^6 PFU/ml of ^{125}I-S. aureus (1 CPM/3 PFU) to "Uno" bronchial plastic catheters (3 cm for each reaction, in duplicate) coated with FN was carried out as described in methods. When competition reaction was performed, the bacteria and the added protein were preincubated at room temperature for 30 minutes and then added to the catheters for further incubation as described in the methods section.

The polypeptides used in the competition r actions were: P-31 (p31 kD), r-20 (recombinant derived 20 kD FBD) and r-31 (reoxidated and refolded r31kD). Some of the reactions (see figure) were measured in the presence of 5 μm Heparin (from porcine intestinal mucosa, molecular weight of 10,000; Sigma).

The binding of "control" reaction in the absence of competitors (8.8% of input bacteria) was normalized to 100%.

Figure 29. Adhesion of p31 kD in Rabbit Aorta Lesion Model

The figure shows the distribution of radioactivity in serially sectioned aorta segments from balloon catheterized rabbits. The measurements were taken 72 hours after injection of ^{125}I-labeled Fibronectin (FN) (Panel A) or plasma derived 31kD FBD (31kD) (Panel B).

Figure 30. Construction of pTV-170

The NdeI-NdeI ApoE fragment was isolated from plasmid pApoE-EX2 (ATCC Accession No. 39787) and inserted into the unique NdeI site of the expression vector p579 (Figure 34) which had been digested with NdeI. The resulting plasmid pTV-170 expresses an analog of natural human ApoE protein having a methionine residue added at the N-terminus.

Figure 31. Construction of pTV-194-80

The ß-lactamase promoter and ribosomal binding site fragment was isolated fr m plasmid pBLA11 (ATCC Accession N . 39788) after digestion with EcoRI and AluI. This fragment was ligated to the large fragment of pTV-170

(Figure 30) plasmid which had b en digest d with NdeI, filled in with DNA polymerase I (Klenow) and then digested with EcoRI.

Figure 32. Recombinant polypeptides of fibronectin domains compared to full-length fibronectin

This figure shows the alignment of cDNA clones encoding various recombinant polypeptides relative to one another and to the full-length sequence of fibronectin cDNA and to a schematic representation of the various domains present within the human fibronectin molecule.

Figure 33. Construction of plasmid pTV301 from plasmids pTV104(2) and p579

Plasmid pTV301 directs the expression of a human growth hormone analog under the control of the λ P_L promoter and the cII ribosomal binding site. The plasmid also contains a T_1T_2 transcription terminator downstream of the cDNA encoding human growth hormone.

Figure 34. Construction of plasmid p579

The rRNA operon T_1T_2 transcription termination fragment was isolated from plasmid pPS1 (deposited with the ATCC under Accession No. 39807) which had been digested with HindIII. The T_1T_2 fragment was inserted into the unique HindIII site of pRO211 (Figure 35) which had been digested with HindIII. The resulting expression vector, p579, contains the λ P_L promoter and the C_{II} ribosomal binding site, followed by the T_1T_2 transcription termination s quences.

Figure 35. Construction of plasmids pRO211 and pRO12

The plasmid pJH200 (deposited with the ATCC under Accession No. 39783) was partially digested with NdeI, treated with DNA polymerase I (Klenow) to fill in the ends and the resulting ends were religated to form the expression vector pRO211. The expression vector pRO211 was digested with NdeI and HindIII, the large fragment isolated and ligated to an NdeI-HindIII bovine growth hormone (bGH) fragment isolated from pAL500 (deposited with the ATCC under Accession No. 39782) to give pRO12. (The NdeI-HindIII fragment was produced from pAL500 by digesting it with EcoRI and ligating to the ends of the digestion product synthetic linkers with the sequence:

TATGGATC
ACCTAGTTAA

The ligation mixture was digested with NdeI and HindIII and the resulting NdeI-HindIII bGH fragment isolated).

Figure 36. Construction of plasmid pFN 196-2 which expresses the r12 kD FBD polypeptide

The large BspMI-HindIII fragment obtained by digestion of plasmid pFN 975-25 (Figure 10) with BspMI and HindIII was ligated by T4 DNA ligase to the synthetic pair of linkers A (see Figure 41). Plasmid pFN 196-2 was produced, transformed into Escherichia coli strain A1645 and retransformed into Escherichia coli strain A4255. Plasmid pFN 196-2 contains the 5'-terminal sequence of fibronectin cDNA from nucleotide 14 to nucleotide 340, i.e., it encodes the first 109 amino acids of the FBD of fibronectin terminating with an arginin residue; it is

not yet known if an additional N-terminal methionine is present in the final polypeptide. Plasmid pFN 196-2 gives good expression of an r12 kD FBD polypeptide under the control of the λ promoter and β-lactamase ribosomal binding site, and has been deposited in the ATCC under Accession No. 68328.

Figure 37. Construction of plasmid pFN 197-10, which expresses a modified 12 kD FBD polypeptide (12 kD')

Plasmid pFN 975-25 was treated as described in Figure 36 except that a different pair of linkers, B (see Figure 41) was used. The ligation produced plasmid pFN 197-10 which encodes the N-terminal sequence of the FBD of FN; however, a modification after nucleotide 340 to produce an NdeI site (CATATG) before the stop codon results in the encoding of a polypeptide containing 111 amino acids where the first 109 amino acids correspond to those of the r12 kD polypeptide followed by two additional amino acid residues, viz. histidine and methionine; it is not yet known if an additional N-terminal methionine residue is present in the final polypeptide. Plasmid pFN ~~975-25~~ 197-10 was transformed into Escherichia coli strain A1645 and hence into Escherichia coli strain A4255, and gave good expression of a modified r12 kD (12 kD') FBD polypeptide under the control of the λ promoter and the β-lactamase ribosomal binding site.

Figure 38. Construction of plasmid pFN 202-5 which expresses r12 kD' FBD fused to the r33 kD cell binding domain (CBD) of fibronectin

The large fragment produced aft r NdeI and HindIII digestion of plasmid pFN 197-10 (Figure 37) was ligated

by T4 DNA ligase to the NdeI-HindIII CBD (cell binding domain) fragment of plasmid pFN 137-2. Plasmid pFN 137-2, deposited in the ATCC under Accession No. 67910 has been described in the parent patent application, U.S. Serial No. 345,952; the r33 kD CBD sequence contains amino acids numbered 1329-1722 of fibronectin (see Figure 1) excluding the 90 amino acids numbered 1600-1689 encoded by the ED-A region (see preface to Brief Description of the Figures).

The resulting plasmid, pFN 202-5, was transformed into Escherichia coli strain A1645 and thence to Escherichia coli strain A4255. Plasmid pFN 202-5 contains the cDNA sequence of the 111 amino acids encoded by plasmid pFN 197-10 followed by the cDNA sequence for r33 kD CBD commencing with the codon for serine (the first amino acid of the r33 kD CBD). This plasmid gave good expression of an approximately 45 kD polypeptide comprising the r12 kD fibrin binding domain and the 33 kD cell binding domain of fibronectin; this fused polypeptide was expressed under the control of the λ promoter and the β-lactamase ribosomal binding site.

Figure 39. Construction of plasmid pFN 195-4 which expresses the r31 kD FBD fused to the sequence DGRGDS

The large fragment obtained by digestion of plasmid pFN 975-25 (Figure 10) with PvuII and HindIII was isolated and ligated with T4 DNA ligase to a pair of synthetic linkers, C (see Figure 41). The resulting plasmid, designated pFN 195-4 was transformed into Escherichia coli strain A1645 and thence to Escherichia coli strain A4255. Plasmid pFN 195-4 contains the full-length FBD cDNA sequence from nucleotide 14 to nucl otide 793

(encoding 260 amino acids) followed by a sequence encoding asp-gly-arg-gly-asp-ser, i.e., the polypeptide encoded has a total of 260 amino acids followed by the sequence DGRGDS; it is not known if an additional N-terminal methionine residue is present in the final polypeptide. Plasmid pFN 195-4 is a good expressor of the r31 kD fibrin binding domain fused to the sequence asp-gly-arg-gly-asp-ser (DGRGDS), under the control of the λ promoter and the ß-lactamase ribosomal binding site.

Figure 40. Construction of plasmid pFN 194-2 which expresses a fused 31 kD FBD-33 kD CBD polypeptide

The large PvuII-HindIII fragment produced by digestion of plasmid pFN 975-25 (Figure 10) with PvuII and HindIII was isolated and ligated with T4 DNA ligase to a pair of linkers, D (Figure 41) and then ligated to the cell binding domain (CBD) fragment obtained by digestion of plasmid pFN 137-2 (ATCC Accession No. 67910) with NdeI and HindIII; see Figure 38 for definition of the CBD domain. The resulting plasmid, designated pFN 194-2, was transformed to Escherichia coli strain A1645 and thence to Escherichia coli strain A4255. Plasmid pFN 194-2 is a low expressor of a fused r31 kD FBD-r33 kD CBD polypeptide of approximate molecular weight 64 kD, under the control of the λ P_L promoter and the ß-lactamase ribosomal binding site. The polypeptide encoded by plasmid pFN 194-2 contains DNA encoding the first 265 amino acids of fibronectin fused to a methionine codon, followed by the cDNA sequence for the CBD of fibronectin, commencing at the codon for amino acid serin at position 1 of the CBD.

Figure 41. Oligonucleotide linkers used in construction of plasmids

Four pairs of chemically synthesized oligomers (A, B, C and D) were prepared and were used to construct plasmids as described in Figures 36, 37, 39 and 40, respectively).

Figure 42. Uptake of labeled r31 kD FBD by stainless steel coil-induced venous thrombi

The bars and vertical brackets represent the mean ± SEM (N=10) of the specific radioactivity associated with isolated thrombi, vein segments carrying the thrombus ("thrombi in-situ"), or peripheral blood samples 24h after administration of ^{125}I-31 kD FBD. For details, see Example 12, Section A.

Figure 43. Comparison of labeled r12 kD, r20 kD and r31 kD FBD polypeptides in the rat venous thrombus model

The bars and vertical brackets represent the mean ± SEM (N=5) of the specific radioactivity associated with isolated thrombi (T) or blood (B) 24 hours after administration of the ^{125}I-labeled recombinant polypeptides, as indicated. For details, see Example 12, Section B.

Figure 44. Metabolic stability of ^{125}I-labeled r31 kD FBD in rats

Rats were injected intravenously with ^{125}I-r31 kD FBD (5 x 10^6 cpm/rat) in a similar experiment to that described in Figur 16. At the time intervals indicated blood samples were removed, plac d in Na citrate c ntaining

tubes (final N-citrate concentration = 0.38%) and blood aliquots resulting were directed as follows: either (a) treated with 20% TCA and the TCA insoluble counts (after TCA precipitation) were measured; or (b) incubated with preformed clot (using 20 µl whole blood from control rat); binding of the ^{125}I-31 kD FBD to the preformed clot was measured under the conditions of the two-step reaction II (Example 11). The radioactivity was measured by a gamma counter and the activity of each sample was calculated as a percentage of total cpm present in the reaction mixture. (Normally TCA precipitation includes placing sample aliquots on filters which are counted for total cpm, washing the filter 3 times with 20% TCA followed by twice with 20% ethanol to extract TCA, and recounting filters for TCA insoluble counts whereby the percentage of TCA-insoluble counts can be calculated.)

Figure 45. Specificity of binding to fibrin: effect of transglutaminase on the binding of r31 kD to a fibrin clot

The binding of ^{14}C-putrescine-r31 kD, ^{125}I-r31 kD FBD and ^{125}I-recombinant growth hormone in the presence or absence of transglutaminase was tested as described in Example 11, B. The radioactivity of the washed fibrin pellet was measured using a ß-counter for the ^{14}C labeling and a gamma counter for the ^{125}I labeling and the ratio of counts in the presence and absence of transglutaminase was calculated for each protein.

Figure 46. Characterization of r31 kD FBD - fibrin complex by SDS polyacrylamide gel electrophoresis

^{125}I-r31 kD FBD was incubated with preformed fibrin clot derived from either 20 µl whole human blood (A) or 250 µl of 0.8 µM solution of human fibrinogen (B). In one of the experiments (B) dental coils were added to the tubes together with the fibrinogen.

Binding of ^{125}I-r31 kD fibrin clot was measured using the two step Reaction II as described in Example 11 using 0.15 µM ^{125}I-r31 FBD in the presence of 5 µM $CaCl_2$. The reaction was terminated by washing three times with PBS and the pellet, after the various treatments described below, was centrifuged. 15 µl aliquots of the supernatant were electrophoresed in SDS-polyacrylamide gels (15% gel). An autoradiogram was developed which is shown in the figure. The tracks of the gel are as follows:

A. 1. plasmatic FN (220 kD)
2. r31 kD FBD
3. r31 kD FBD incubated for 1 hour with 2 units/ml plasmin (Sigma)

4-6. Binding experiments with rFBD incubated with preformed fibrin clot derived from citrated whole blood.

4. ^{125}I-r31 kD - fibrin pellet was boiled in sample buffer for 10 minutes (Sample buffer = 0.7 M ß-mercapto thanol/3% SDS/60 mM tris HCl, pH 6.8).

5. Binding conditions as described above with the addition of 0.02 units/ml guinea-pig liver transglutaminase (Sigma); pellet treated as in 4.

6. Binding conditions as described in (5); the pellet was incubated with 2 units/ml plasmin for 1 hour.

B. 1-3. Experiments with r31 kD FBD incubated with preformed fibrin clot derived from pure human fibrinogen in the presence of transglutaminase.

1. Boiling of ^{125}I-r31 kD - FBD - fibrin pellets in sample buffer.

2. Boiling of ^{125}I-r31 kD - FBD - fibrin pellets in phosphate - saline buffer.

3. Boiling of ^{125}I-r31 kD - FBD - fibrin pellets in sample buffer plus 4 M urea.

4-5. Experiments as B. 1-3 above, but clots were formed in the presence of dental coils. Coils containing the clots were removed to different tubes and then the binding of ^{125}I-r31 kD to the clot was measured. The binding reaction was terminated by removal of the coils and analysis of the ^{125}I-r31 kD - fibrin complex attached to the coils.

4. Boiling of ^{125}I-r31 kD - FBD - fibrin pellet in sample buffer.

5. Boiling of ^{125}I-r31 kD - FBD - fibrin pellet in sample buffer plus 4 M urea.

Figure 47. Binding of ^{125}I-r31 kD FBD to preformed fibrin clots: effect of fibronectin (FN) and heparin

Experiments A and B were performed essentially as described for the two step Reaction II in Example 11.

A. Fibrin clots were formed during a 45 minute incubation period using 20 µl citrated whole blood, 5 mM $CaCl_2$, 1 unit/ml thrombin in a final volume of 250 µl. The fibrin pellets, after centrifugation, were placed in 200 µl PBS - 5 mM $CaCl_2$ solution with or without purified plasma-derived FN (1 µM) and ^{125}I-r31 kD FBD (0.15 µM, specific activity 4 x 10^5 cpm/µg). The binding of ^{125}I-r31 kD was determined after an additional incubation period of 30 minutes by measurement of radioactivity by a gamma counter.

B. Fibrin clots were formed as indicated in A. At the end of the first incubation period, ^{125}I-r31 kD FBD (0.15 µM, specific activity 3 x 10^4 cpm/µg) was added together with various concentrations of heparin of average molecular weight 10,000 daltons (Laboratoire Choay, Paris, France; stock solution 5000 i.u./ml); the incubation was continued for an additional 30 minutes. The binding of ^{125}I-r31 kD was then determined by measurement of radioactivity by a gamma counter.

Figure 48. Binding of fibrin binding domain polypeptides to the fibrin clot

This experiment was carried out essentially as described for the two-step Reaction II (Example 11). 0.15 μM ^{125}-I of one of the fibrin binding domain polypeptides as indicated below was incubated at 37°C with preformed fibrin clot derived from 20 μl citrated whole blood. The binding was measured in the presence of 5 mM $CaCl_2$ and 0.02 units/ml transglutaminase. The reaction was terminated, after a 45 minute incubation, by centrifugation; the pellet was washed three times with PBS and the radioactivity was measured in a gamma counter.

1. plasmatic 31 kD FBD (p31 kD)
2. r12 kD
3. r20 kD
4. r31 kD (Batch A)
5. r31 kD (Batch B)
6. r31 kD (Batch C)

Figure 49. Comparison of binding of ^{125}I-r12 kD to fresh and frozen fibrin clots

This experiment was carried out essentially as described for the two-step Reaction II (Example 11). Preformed fibrin clots derived from 20 μl citrated whole human blood were either frozen at -70°C for 7 days (frozen clots) or used immediately after their formation (fresh clots).

The fibrin clots were incubated with 0.15 μM ^{125}I-r12 kD in the presence or absence of 0.02 units/ml guinea-pig

liver transglutaminase (Sigma). The binding to fibrin clots was measured as described in Example 11.

Figure 50. Conditions for binding of ^{125}I-r31 kD FBD to preformed clots

This experiment was carried out essentially as described for the two-step Reaction II (Example 11).

The fibrin clot was produced in citrated blood (A) and "naive" blood (B). The clots were incubated for the binding step at 37°C in a final volume of 250 µl PBS with 0.15 µM ^{125}I-rFBD and other constituents as indicated in the figure and below. The concentrations given are the final concentration in the reaction mixture.

1. Control (no added constituents)
2. 5 mM $CaCl_2$
3. 5 mM $CaCl_2$/ 2 units/ml hirudin (Sigma)
4. 5 mM $CaCl_2$/ 2 units/ml hirudin/ 0.02 units/ml transglutaminase
5. 5 mM $CaCl_2$/ 0.02 units/ml transglutaminase
6. 0.02 units/ml transglutaminase

(All the exogenous transglutaminase used in the experiments described in this application was guinea-pig liver transglutaminase from Sigma).

Figure 51. Binding of ^{125}I-r31 kD FBD to ECM in the presence of various constituents

0.3 µM of ^{125}I-r31 kD FBD was incubated with extracellular matrix of endothelial cells (ECM) as described by Eld r et al. (20) in a 37°C CO_2-incubator in the presence of saline, 3 mM $CaCl_2$ and 0.02 units/ml of

guinea-pig liver transglutaminase (TG) and other constituents as indicated in the figure. The control was done in the absence of transglutaminase.

The reactions were terminated after 45 minutes by washing five times with PBS. Plates were extracted with a solution of 0.5% SDS and aliquots of the extracted material were measured in a gamma counter.

Figure 52. Refolding and purification of the r20 kD polypeptide as monitored by elution profiles from a Superose 12 column (attached to a FPLC)

Aliquots of 200 µl of the r20 kD polypeptide at various stages of the refolding and purification process were injected on top of a Superose 12 column (attached to a FPLC). The column was equilibrated and eluted with a solution of 150 mM NaCl/20 mM Tris HCl, pH 7.8, at a flow rate of 0.8 ml/min. The lower trace is from the FPLC Controller LCC-500. A. Pellet of r20 kD polypeptide solubilized in 6 M Guanidine-HCl and reduced with 50 mM ß-mercaptoethanol; B. Refolded and air-reoxidized r20 kD polypeptide; C. Q-Sepharose bound polypeptides, i.e., material which was separated from the purified r20 kD; D. Flow-through from the Q-Sepharose column; E. Flow-through from the Heparin-Sepharose column, i.e., material which was separated from the purified r20 kD; F. Purified 20 kD polypeptide (retention time = 18.16 min), eluted from the Heparin-Sepharose column with 0.5 M NaCl. Note that there is no peak at this retention time of 18.16 min in Profile A, where the material is in reduced form, nor in Profiles C & E, which contain incorrectly folded forms of the 20 kD polypeptide.

Figure 53. Refolding and purification of the r12 kD polypeptide as monitored by elution profiles from a Superose 12 column (attached to a Waters HPLC system)

Aliquots of 25-100 µl of the r12 kD polypeptide at various stages of the refolding and purification process were injected on top of a Superose 12 column (attached to a Waters HPLC system). The column was equilibrated and eluted with a solution of 150 mM NaCl/20 mM Tris HCl, pH 7.8, at a flow rate of 0.8 ml/min. A. Pellet of r12 kD polypeptide solubilized in 6 M Guanidine-HCl and reduced with 50 mM ß-mercaptoethanol; B. Refolded and air-reoxidized r12 kD polypeptide; C. Q-Sepharose bound polypeptides, i.e., material which was separated from the purified r12 kD; D. Flow-through from both the Q- and Heparin-Sepharose columns (in this case, the columns were connected in series and the flow-through from the Q-Sepharose was therefore automatically loaded on the Heparin-Sepharose column), i.e., material which was separated from the purified r12 kD; E. Purified r12 kD polypeptide (retention time - 18.83 min), eluted from the Heparin-Sepharose column with 0.5 M NaCl. Note that there is no peak at this retention time of 18.83 min in Profile A, where the material is in reduced form, nor in Profiles C & D, which contain incorrectly folded forms of the r12 kD polypeptide.

DETAILED DESCRIPTION OF THE INVENTION

The plasmids pFN 975-25, pFN 949-2, pFN 137-2, and pFN 196-2 were deposited pursuant to, and in satisfaction of, the requirements of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852 under ATCC Accession Nos. 67832, 67831, 67910, and 68328, respectively. Similarly, many of the other ATCC deposits referred to in the subject application were also deposited pursuant to, and in satisfaction of, the requirements of the Budapest Treaty.

The fibrin binding domain of plasma fibronectin consists of five repetitive finger-like loops of 41-52 amino acids in length. Each loop has two adjacent intra-loop disulfide bonds, i.e. 4 cysteine residues per loop. This repeat structure is termed Type I homology (8).

The recombinant fibrin binding domain (FBD) polypeptides described in this application comprise either the full length fibrin binding domain (the r31 kD polypeptide) or smaller proteins (r20 kD and r12 kD polypeptides) of the fibrin binding domain. These smaller polypeptides are smaller than 31 kD and comprise part of the sequence of the fibrin binding domain. Many other polypeptides of the fibrin binding domain may be expressed by additional plasmids constructed, using methods known in the art, from plasmids described in this application and these polypeptides may be refolded, reoxidized, and purified using m thods describ d in this application.

The full length recombinant fibrin binding domain (the r31 kD polypeptide) described in this application comprises the first 262 amino acids of fibronectin which the sequence arg-ala-ala-val at the carboxy-terminus. It is not yet known if an additional methionine residue is present at the amino terminus of the final polypeptide. The plasmatic fibrin binding domain derived by tryptic digestion of plasma fibronectin comprises the first 259 amino acids of fibronectin, i.e. with arginine at the carboxy-terminus.

The r31 kD polypeptide has five of the Type I homology loops discussed above (i.e. 10 disulfide bonds), the r20 kD polypeptide has three loops (i.e. 6 disulfide bonds), and the r12 kD polypeptide has two loops (i.e. 4 disulfide bonds). The presence of these disulfide bonds explains the necessity and also the difficulty of the refolding/reoxidation procedure developed to obtain and purify correctly folded FBD polypeptides which have the correct disulfide bonds. The correctly folded FBD polypeptides are biologically active, i.e. they can bind to fibrin and/or to Staphylococcus aureus.

The recombinant FBD polypeptides are produced in inclusion bodies which are contained in the pellet produced after disruption of the cell cake.

This invention discloses the production of recombinant fibronectin fibrin binding domain (FBD) polypeptides for use in thrombus imaging, prevention of thrombus formation, and prevention of bacterial infection. These polypeptides may also be bound to a thrombolytic agent for targeting the agent to a thrombus.

The recombinant cells which produce the FBD polypeptides can be any cells in which a DNA sequence encoding an FBD polypeptide has been introduced by recombinant DNA techniques. The cell must be capable of expressing the DNA sequence and producing the polypeptide product. The cell may be a mammalian cell, a fungal cell such as a yeast cell, or a bacterial cell.

The bacterial cell can be any strain including auxotrophic, prototrophic and lytic strains, F^+ and F^- strains, strains harboring the cI857 repressor sequence of the λ prophage and strains deleted for the deo repressors or the deo gene.

Examples of wild type Escherichia coli strains are prototroph ATCC No. 12435, and auxotroph MC1061 (ATCC Accession No. 67361).

Examples of Escherichia coli strains which harbor the λ cI857 repressor sequence are the auxotrophs A1645 harboring plasmid pTVR 279-8 (ATCC No. 53216), A1637 harboring plasmid pTV 104(2) (ATCC No. 39384), and A2097 harboring plasmid pSODα2 (ATCC No. 39786), and the prototrophs A4255 harboring plasmid pFN 975-25 (ATCC No. 67832) and biotin-independent A4346 harboring plasmid pHG44 (ATCC No. 53218).

An example of a lytic Escherichia coli strain is A4048 which harbors plasmid pHG44 (ATCC No. 53217).

Examples of F^- strains are Escherichia coli sφ930 (F^-) harboring plasmid pMF 5534 deposited under ATCC No. 67703 and Escherichia coli W31100 (F^-) harboring plasmid pEFF 920 deposited under ATCC No. 67706.

Examples of Escherichia coli strains deleted for the deo gene or deo repressors are Sφ732 harboring plasmid pMF 2005 (ATCC No. 67362), Sφ540 harboring plasmid pJBF 5401 (ATCC No. 67359), and Sφ930 harboring plasmid pEFF 920 (ATCC No. 67706) (see European Patent Application Publication No. 0303972, published February 22, 1989).

The plasmids of this invention may be introduced into suitable bacterial host cells, preferably Escherichia coli. An example of a suitable Escherichia coli cell is strain A4255 (F') [ATCC Accession No. 67832], but other Escherichia coli strains and other bacteria can also be used as host cells for the plasmids. Such bacteria include Pseudomonas aeruginosa and Bacillus subtilis.

All of the Escherichia coli host strains described above can be "cured" of the plasmids they harbor by methods well known in the art, e.g. the ethidium bromide methods described by R.P. Novick in Bacteriol. Review 33: 210 (1969).

The bacterial cell may contain the FBD sequence encoding the FBD polypeptide in the body of a vector DNA molecule such as a plasmid. The vector or plasmid is constructed by recombinant DNA techniques so that the sequence encoding the FBD polypeptide is incorporated at a suitable position in the molecule.

Plasmids used for production of the FBD polypeptides can harbor a variety of promoters such as the λ promoter or the deo promoters.

Among the plasmids which may b used for production of FBD polypeptides are the following:

a) Plasmid pFN 975-25 which expresses the r31 kD FBD and which has been deposited in Escherichia coli strain A4255 in the ATCC under Accession No. 67832;

b) Plasmid pFN 949-2 which expresses the r20 kD FBD and which has been deposited in Escherichia coli strain A4255 in the ATCC under Accession No. 67831;

c) Plasmid pFN 196-2 which expresses the r12 kD FBD and which has been deposited in Escherichia coli strain A4255 in the ATCC under Accession No. 68328;

d) Plasmid pFN 197-10 which expresses a modified 12 kD FBD polypeptide, and which has been described in Figure 37 of this application;

e) Plasmid pFN 195-4 which expresses the r31 kD polypeptide fused to the sequence DGRGDS, and which has been described in Figure 39 of this application;

f) Any plasmid, derived from the above plasmids, containing FBD sequences encoded by the above plasmids; and

g) Any plasmid which contains FBD sequences encoded by the above plasmids.

The subject invention provides an imaging agent which comprises a polypeptide labeled with an imageable marker, such polypeptid having an amino acid sequence substantially pr s nt in th fibrin binding domain of

naturally-occurring human fibronectin and being capable of binding to fibin. Also provided is a composition comprising an effective imaging amount of such an imaging agent and a physiologically acceptable carrier.

The polypeptides which are labeled with an imageable marker may be, i.e., fragments of the fibrin binding domain of human fibronectin; they may be produced using recombinant DNA techniques; or they may be synthesized in a DNA synthesizer. Applicants have provided three examples of such polypeptides, with the preferred embodiment being the 12 kD polypeptide. As would be understood by one skilled in the art, the terms "having an amino acid sequence substantially present in the fibrin binding domain of naturally-occurring human fibronectin" encompasses, i.e., naturally-occurring allelic variations and recombinant variations, such as site-directed mutagenesis. These are all encompassed by applicants' "polypeptide", the only limitation being the ability to bind to fibrin.

The imageable marker used is a matter of choice to one skilled in the art. It is preferred that the marker be a radioactive isotope, an element which is opaque to X-rays, or a paramagnetic ion.

Radioactive isotopes are commonly used in medicine and are well known to those skilled in the art. It is presently preferred that the marker be indium-111, technetium-99m, iodine-123, iodine-125, iodine-131, krypton-81m, xenon-133, or gallium-67, or mixtures thereof. Most preferably, the marker is technetium or indium.

The d tectable marker may also be a paramagnetic ion. Paramagnetic ions are also commonly used in medicine. Examples of such markers include chelated metal ions of chromium (III), manganese (II), iron (III), iron (II), cobalt (II), nickel (II), copper (II), praseodymium (III), neodymium (III), samarium (III), gadolinium (III), terbium (III), dysprosium (III), holmium (III), erbium (III), ytterbium (III), or mixtures thereof.

Preferably, the imaging agent comprises a polypeptide which is a 31 kD polypeptide corresponding to an amino acid sequence present in the fibrin binding domain and having the amino acid sequence of amino acids 1-262 as shown in Figure 1, i.e. the full length, of the fibrin binding domain of human fibronectin; a 20 kD polypeptide corresponding to an amino acid sequence present in the fibrin binding domain of human fibronectin and having the amino acid sequence of amino acids 1-153 as shown in Figure 1; the 20 kD polypeptide comprising less than about 20 additional amino acids; or a 12 kD polypeptide corresponding to an amino acid sequence present in the fibrin binding domain of human fibronectin and having the amino acid sequence of amino acids 1-109 as shown in Figure 1.

The subject invention also provides a method for imaging a fibrin-containing substance, i.e. a thrombus or atherosclerotic plaque, which comprises contacting the fibrin-containing substance to be imaged with the agent as disclosed above under conditions such that the agent binds to the fibrin-containing substance and imaging bound agent and thereby imaging the fibrin-containing substance.

Further provided is a method f r imaging a fibrin-containing substance in a subject which comprises:

(a) administering to the subject a composition of the agent as disclosed above under conditions permitting the imaging agent therein to enter the blood stream and bind to fibrin present in the blood vessels;

(b) imaging bound agent within the blood vessels; and thereby

(c) imaging the fibrin-containing substance.

Preferably, the polypeptide of the reagent used in the above methods for imaging a fibrin-containing substance is a 31 kD polypeptide corresponding to an amino acid sequence present in the fibrin binding domain and having the amino acid sequence of amino acids 1-262 as shown in Figure 1, i.e. the full length, of the fibrin binding domain of human fibronectin; a 20 kD polypeptide corresponding to an amino acid sequence present in the fibrin binding domain of human fibronectin and having the amino acid sequence of amino acids 1-153 as shown in Figure 1; the 20 kD polypeptide comprising less than about 20 additional amino acids; or a 12 kD polypeptide corresponding to an amino acid sequence present in the fibrin binding domain of human fibronectin and having the amino acid sequence of amino acids 1-109 as shown in Figure 1.

Preferred markers used in the above methods for imaging a fibrin-containing substance are radioactive isotopes, elements which are opaque to X-rays, or paramagnetic ions. Most preferred markers ar radioactive isotopes,

such as indium-111, technetium-99m, iodin -123, iodine-125, iodine-131, krypton-81m, xenon-133, and gallium-67.

Imaging may be done through any of the methods known to one skilled in the art. These methods include but are not limited to X-ray, CAT scan, PET scan, NMRI, and fluoroscopy. Preferably, the imaging of the fibrin-containing substance by the above methods is carried out using a gamma camera.

Further provided is a plasmid for expression of a polypeptide having an amino acid sequence substantially present in the fibrin binding domain of naturally-occurring human fibronectin and being capable of binding to fibrin comprising DNA encoding the polypeptide and DNA encoding suitable regulatory elements positioned relative to the DNA encoding the polypeptide so as to effect expression of the polypeptide in a suitable host cell.

Applicants have provided four examples of fibrin binding domain polypeptides. These include the p31 kD, r31 kD, r20 kD, and r12 kD polypeptides. These polypeptides exhibit the binding and adhesive properties of portions of naturally-occurring human fibronectin. The scope of the claims of the subject application are not intended to be limited to these four FBD polypeptides, which are examples of preferred embodiments only.

Three forms of the r31 kD fibrin binding domain polypeptide are defined in Example 5 as:

(a) the polypeptide as it is obtained from a washed pellet, after dissolution in 6 M GuCl, i.e. in "scrambled" form;

(b) the fully reduced polypeptide, present after treatment with a reducing agent such as GSH in the presence of 6 M GuCl; and

(c) the reoxidized-refolded polypeptide, obtained by treatment with the GSH/GSSG as described in Example 5.

The "scrambled" r31 kD polypeptide is apparently improperly folded due to the formation of one or more incorrect disulfide bonds.

Unless otherwise stated, the r31 kD polypeptide described is correctly folded, i.e. form (c). Similarly, the 20 kD and 12 kD polypeptides may also occur in these three forms but the 20 kD and 12 kD polypeptides used in the experiments are correctly folded.

In preferred embodiments, the polypeptide is about a 31 kD polypeptide corresponding to an amino acid sequence present in the fibrin binding domain of human fibronectin; about a 20 kD polypeptide corresponding to an amino acid sequence present in the fibrin binding domain of human fibronectin; or about a 12 kD polypeptide corresponding to an amino acid sequence present in the fibrin binding domain of human fibronectin.

In more preferred embodiments, the polypeptide is a 31 kD polypeptide corresponding to an amino acid sequence present in the fibrin binding domain and having the amino acid sequence of amino acids 1-262 as shown in Figure 1, i.e. the full length, of the fibrin binding domain of human fibronectin; a 20 kD polypeptide corresponding to an amin acid sequence present in the fibrin binding domain of human fibronectin and having th amino acid

sequence of amino acids 1-153 as shown in Figure 1; the 20 kD polypeptide comprising less than about 20 additional amino acids; or a 12 kD polypeptide corresponding to an amino acid sequence present in the fibrin binding domain of human fibronectin and having the amino acid sequence of amino acids 1-109 as shown in Figure 1.

Naturally-occurring human fibronectin is as it occurs in the human body (in plasma).

As used throughout this application, a substantial portion is at least one fifth (1/5). A polypeptide which has the biological activity of the fibrin binding domain of naturally-occurring human fibronectin exhibits binding or adhesive properties similar to the fibrin binding domain of naturally-occurring human fibronectin when the level of such activity is assayed or determined.

In this invention, the amino acid sequence of the various functional domains are determined by cleavage of the cDNA which encodes the domains with restriction enzymes, and do not necessarily correspond to the amino acid sequence of the domains as obtained and defined by proteolytic digestion of fibronectin.

The plasmid of this invention further comprises suitable regulatory elements positioned relative to the DNA encoding the polypeptide so as to effect expression of the polypeptide in a suitable host cell, such as promoters and operators, e.g. λ P_LO_L, ribosomal binding sites, e.g. C_{II}, and repressors. Other suitable regulatory elements include, for example, the lac, trp, tac, lpp and deo promoters (European Patent Application Publication No. 0303972, published February 22, 1989).

The suitable regulatory elements ar positioned r lative to the DNA encoding the polypeptide so as to effect expression of the polypeptide in a suitable bacterial host cell. In preferred embodiments of the invention, the regulatory elements are positioned close to and upstream of the DNA encoding the polypeptide.

The invention provides a plasmid designated pFN 975-25 and deposited in Escherichia coli strain A4255 (F^+) under ATCC Accession No. 67832. Plasmid pFN 975-25 encodes a 31 kD polypeptide of the fibrin binding domain of human fibronectin comprising amino acids 1-262.

Further provided is a plasmid designated pFN 949-2 and deposited in Escherichia coli strain A1645 under ATCC Accession No. 67831. Plasmid pFN 949-2 encodes a 20 kD polypeptide of the fibrin binding domain of human fibronectin comprising amino acids 1-153 and less than 20 additional amino acids.

Also provided is a plasmid designated pFN 196-2 and deposited in Escherichia coli strain A4255 under ATCC Accession No. 68328. Plasmid pFN 196-2 encodes a 12 kD polypeptide of the fibrin binding domain of human fibronectin comprising amino acids 1-109.

In presently preferred embodiments, the invention provides an Escherichia coli cell containing the plasmid designated pFN 975-25 and wherein the cell is deposited under ATCC Accession No. 67832; an Escherichia coli cell containing the plasmid designated pFN 949-2 and wherein the cell is deposited under ATCC Accession No. 67831; and an Escherichia coli cell containing the plasmid d signated pFN 196-2 and wherein the cell is deposited und r ATCC Accession No. 68328.

The invention provides a method of producing a polypeptide having an amino acid sequence substantially present in the fibrin binding domain of naturally-occurring human fibronectin and being capable of binding to fibrin which comprises treating a cell containing a plasmid comprising DNA encoding the polypeptide so that the DNA directs expression of the polypeptide and recovering from the cell the polypeptide so expressed.

Preferably, the polypeptide so produced is a 31 kD, 20 kD, or 12 kD polypeptide of the fibrin binding domain.

Further provided is a purified polypeptide substantially free of other substances of human origin which has an amino acid sequence substantially present in the fibrin binding domain of naturally-occurring human fibronectin and being capable of binding to fibrin.

Preferably, the polypeptide is a 31 kD polypeptide corresponding to an amino acid sequence present in the fibrin binding domain and having the amino acid sequence of amino acids 1-262 as shown in Figure 1, i.e. the full length, of the fibrin binding domain of human fibronectin; a 20 kD polypeptide corresponding to an amino acid sequence present in the fibrin binding domain of human fibronectin and having the amino acid sequence of amino acids 1-153 as shown in Figure 1; the 20 kD polypeptide comprising less than about 20 additional amino acids; or a 12 kD polypeptide corresponding to an amino acid sequence present in the fibrin binding domain of human fibronectin and having the amino acid sequence of amino acids 1-109 as shown in Figure 1.

The invention further provides such a purified polypeptide substantially free of other substances of

human origin fused to a second polypeptide, the second polypeptide comprising a substantial portion of the amino acid sequence of the cell binding domain of naturally-occurring human fibronectin.

Preferably, the fused polypeptide is a 45 kD fused polypeptide, wherein the purified polypeptide is a 12 kD polypeptide and the second polypeptide which comprises a substantial portion of the cell binding domain of naturally-occurring human fibronectin is a 33 kD polypeptide. The fused polypeptide may also comprise a 31 kD purified polypeptide and a second polypeptide which contains the amino acid sequence GRGDS. Another preferred fused polypeptide is a 64 kD fused polypeptide, wherein the purified polypeptide is a 31 kD polypeptide and the second polypeptide which comprises a substantial portion of the cell binding domain of naturally-occurring human fibronectin is a 33 kD polypeptide.

The invention also provides a plasmid for expression of the 45 kD fused polypeptide, disclosed above, designated pFN 202-5; a plasmid for expression of the 31 kD/GRGDS fused polypeptide, disclosed above, designated pFN 195-4; and a plasmid for expression of the 64 kD fused polypeptide, disclosed above, designated pFN 194-2.

As used throughout the subject application, "fused" or "bound" encompasses polypeptides bound covalently, non-covalently, or conjugated. The polypeptides may be conjugated through other chemical moities including amino acid or polypeptide cross-linkers, which are standardly used in the art and are well-known to those skilled in the art to which the subject invention pertains.

This invention further provides a m thod of treating a subject susceptible to, or afflicted with, a bacterial infection which comprises administering to the subject an amount of any of the disclosed polypeptides effective to prevent or treat the bacterial infection. The susceptibility to bacterial infection may be due to the presence of a catheter or an implant in the subject.

Numerous methods are known in the art for detection of thrombi, such as radioactive labeling (nuclear medicine use of isotopes), radio-opaque labeling (such as CAT scan), and Magnetic Resonance Imaging (MRI). Any of these labeling methods can be used in the method of the subject invention for detecting the thrombus. In each of these detection methods the polypeptide is used as a diagnostic agent for detecting the thrombus.

The invention provides a coated medical device comprising a medical device and the polypeptides of the fibrin binding domain of naturally-occurring human fibronectin applied as a coating to the surface of the medical device. Examples of medical devices which may be coated include catheters, medical implants (such a hip replacement and prostheses), tubings and syringes.

The invention provides a method of minimizing risk of bacterial infection associated with use of medical devices, preferably a catheter, which comprises:

(a) applying the polypeptide of the fibrin binding domain of fibronectin as a coating to a surface of the device; and

(b) employing th resulting coated devic rather than an uncoated device.

The invention also provides a method of minimizing risk of bacterial infection associated with the use of medical devices which comprises employing a device coated with the polypeptides disclosed in the subject application rather than an uncoated device.

Also provided is a method of refolding and reoxidizing a polypeptide having an amino acid sequence substantially present in the fibrin binding domain of naturally-occurring human fibronectin and being capable of binding to fibrin which comprises contacting the polypeptide with a thiol-containing compound and a disulfide so as to refold and reoxidize the polypeptide.

Preferably, the thiol-containing compound is selected from the group consisting of glutathione, thioredoxin, ß-mercaptoethanol, and cysteine.

Preferably, the thiol-containing compound is ß-mercapto-ethanol and the disulfide is produced <u>in situ</u> by introduction of air.

Preferably, the polypeptide is selected from the group consisting of a 31 kD polypeptide, a 20 kD polypeptide and a 12 kD polypeptide.

The method of refolding and reoxidizing may additionally comprise contacting the polypeptide with a denaturant. Preferred denaturants are guanidine hydrochloride and urea.

Preferably, the polypeptide is at a low concentration, such as below 600 µg/ml.

The subject invention also provides a method for recovering a purified biologically active polypeptide having an amino acid sequence substantially present in the fibrin binding domain of naturally-occurring human fibronectin and being capable of binding to fibrin from a cell in which the polypeptide has been produced by means of expression of a plasmid containing DNA encoding the polypeptide which comprises:

(a) disrupting the cell so as to produce a lysate containing the polypeptide;

(b) centrifuging the lysate so as to concentrate the polypeptide;

(c) separating the concentrated polypeptide;

(d) solubilizing the separated, concentrated polypeptide;

(e) refolding and reoxidizing the solubilized polypeptide;

(f) separating the refolded and reoxidized polypeptide; and

(g) recovering the purified, refolded and reoxidized polypeptide.

Preferably, the refolding and reoxidizing comprises contacting the polypeptide with a thiol-containing compound and a disulfide so as to refold and reoxidize the polypeptide. Preferably, th thiol-containing compound is selected from the group consisting f

glutathi ne, thioredoxin, ß-mercaptoethanol, and cysteine.

In one preferred embodiment, the thiol-containing compound is ß-mercaptoethanol and the disulfide is produced <u>in situ</u> by introduction of air.

Preferably, the polypeptide is selected from the group consisting of a 31 kD polypeptide, a 20 kD polypeptide and a 12 kD polypeptide.

The method may additionally comprises contacting the polypeptide with a denaturant, such as guanidine hydrochloride or urea.

Preferably, the polypeptide is at a low concentration, such as below 600 µg/ml.

Preferably, the separating of the concentrated polypeptide in step (c) comprises chromatography, preferably Heparin-Sepharose chromatography.

The subject invention also provides a method of inhibiting thrombus formation in a subject susceptible to thrombus formation which comprises administering to the subject an amount of a polypeptide (selected from the polypeptides and fused polypeptides disclosed above) effective to inhibit thrombus formation. The polypeptide may be reduced or alternatively the S-H groups may be blocked (e.g. by carboxyamidation to prevent reoxidation).

The subject invention also provides a polypeptide as disclosed ab ve bound to a thr mbolytic agent for targ ting of thrombolytic agents. The thrombolytic

agents may be selected from tissue plasminogen activator (TPA), urokinase, streptokinase, prourokinase, Anisoylated Plasminogen-Streptokinase Activator Complex (Eminase™), TPA analogs, or a protease.

Further provided is a method for achieving thrombolysis of a thrombus which comprises administering to a subject an amount of the polypeptide bound to a thrombolytic agent effective to achieve thrombolysis.

EXAMPLES

All the references to map positions correspond to the identically numbered positions along the nucleotide sequence of human fibronectin cDNA shown in Figure 1 (see also Figure 3 of Baralle, F.E., European Patent Publication No. 207,751, published January 7, 1987).

This patent application is directed to polypeptides of the N-terminus fibrin binding domain (FBD). Some of the proteins described are fusion proteins comprising an FBD fragment joined at its C-terminus to a fragment of the cell binding domain (CBD).

The cDNA sequence corresponding to the CBD which applicants have cloned and expressed is missing the 270 bp extra domain (ED) segment which extends from nucleotides 4812 to 5081, inclusive, on the Baralle map (see Figure 1). Thus, the cDNA sequence which is said to extend from nucleotide 3317 to 5566 on the Baralle map, contains only 1980 nucleotides, because it is missing the 270 nucleotides of the ED segment, namely from nucleotides 4812 to 5081 inclusive; this region is also known in the art as the ED-A region; concomitantly amino acid 1690 is changed from alanine to threonine. Similarly, the polypeptide expressed by that DNA fragment would encode from amino acid 1102 to amino acid 1851 on the Baralle map but would be missing the 90 amino acids encoded by the ED region, namely amino acids 1600-1689 inclusive, and thus it would contain only 660 amino acids. This is true for all CBD fragments described in this application which span the ED region. (The region known in the art as the ED-B region is missing both in Baralle's sequence and in applicants' cDNA.)

The EcoRI cleavag site shown at position 3317 was constructed by applicants during the cloning procedure by use of EcoRI linkers. This GAATTC sequence at positions 3313 to 3318 differs in 1 nucleotide from the corresponding Baralle sequence GATTC. This introduces a single nucleotide change C to A at nucleotide 3315. This changes the corresponding amino acid from Thr to Asn.

EXAMPLE 1

Preparation of a Fibronectin cDNA Library

A cDNA library was prepared in λgt11 from poly A+ mRNA isolated from human liver according to the published procedures (13,14). The cDNA fragments were cloned using EcoRI linkers and the cDNA library was screened for fibronectin (FN) positive plasmids using the following synthetic DNA probes.

Probes for cell binding domain (CBD):

Probe	Nucleotides
(3')CACTCTATAATGTCCTAGTGAATGCCTCTTTGTCCTCC	(4355-4392)
(3')AGAATCTCCTTCTGTCTTTTGTCCAGAACTAAG	(3967-3999)
(3')CCGGTTGTTAGTTGTCAAAGACTACAAGGCTCCCTGGACC	(4200-4239)

Probes for N-terminal fibrin binding domain (FBD):

(3')GGGGGTCGGAGGGATACCGGTGACACAGTGTCTTAA	(817-850)
(3')CGACGGGTGCTCCTTTAGACGTGTTGGTTACTTCCCCAGTAC	(1310-1340)

A series of FN cDNA clones covering the entire region of fibrin, collag n, heparin and cell binding domains was identified and isolated (Figure 32). The cDNA fragments w re subcl ned into the Ec RI site of pBR322.

The mRNA f FN is alternatively spliced and therefore different length cDNA's have been reported in the literature. Applicants' cDNA corresponding to the cell binding domain has a 270 base pair deletion from base 4811 to base 5080 on the FN physical map (the complete non spliced cDNA).

EXAMPLE 2

Expression and Purification of Fibrin Binding Domain (FBD) Polypeptides

A. Expression of a partial FBD 20 kD polypeptide

The FN cDNA clones obtained as described in Example 1 and depicted in Figure 32, did not include DNA encoding amino acids 1-190 of the FN molecule. These amino acids are part of the FBD. The DNA corresponding to nucleotides 14 to 472 and coding for amino acids 1-153 (Figure 1A) was constructed by ligation of 7 pairs of chemically synthesized nucleotides (Figures 2A, 2B and 3). The synthetic DNA fragment was designed to contain an ATG initiation codon at the 5' end as well as convenient restriction sites for introduction into various expression vectors. To enable further manipulation of the DNA sequence coding for the FBD, nucleotide number 19, thymidine (T) was changed to adenine (A), thereby eliminating a DdeI restriction site without altering the amino acid sequence. (The site of the nucleotide change is denoted by an asterisk in linker #1 shown in Figure 2A.) The various steps for the cloning of the above synthetic DNA fragment into pBR322 plasmid vector digested with EcoRI and BamHI are described in Figure 3. The plasmid obtained was designated pFN 932-18. The DNA fragment coding for the first 153 N-terminal amino acids of fibronectin from plasmid pFN 932-18, was inserted into pTV 301, a λ P_L expression vector, between the NdeI and BglII sites replacing the DNA sequence coding for human growth hormone (hGH) in plasmid pTV 301 (Figure 4).

The resulting plasmid, pFN 949-2, was deposited with the American Type Culture C llection under Accession No.

67831. Plasmid pFN 949-2 was used to transform Escherichia coli prctotroph A4255. These transformed Escherichia coli cells were found to express the partial FBD polypeptide in amounts comprising about 5% of the total cellular proteins. The polypeptide has a mobility of about 20 kD on reduced SDS polyacrylamide gels as determined from the mobility of the size markers. The polypeptide comprises the first 153 amino acids of fibronectin followed by 4 amino acids coded for by a synthetic linker and then several amino acids resulting from readthrough into the pBR322 vector, i.e., a total of 153 amino acids plus less than 20 additional amino acids, if the additional N-terminal methionine is present. Throughout this specification the polypeptide is referred to as the r20 kD polypeptide or the r20 kD FBD.

B. Expression of a "complete" FBD polypeptide

In order to obtain expression of the entire FBD polypeptide containing amino acids 1 to 262 the following plasmids were constructed:

1. Insertion of termination codon TAA at the 3' end

A synthetic oligonucleotide containing a TAA termination codon and a BglII site having the following sequence:

5' CTGTTTAATAAGCA
3' GACAAATTCGTCTAG

was ligated to the 3' end of an EcoRI-PvuII fragment isolated from FN cDNA clone p931-5 and to a pBR322 vector digested with EcoRI and BamHI as described in Figur 5. The plasmid obtained was designated pFN935-12.

2. Subcloning of carboxy terminal region of FBD in a λ P_L expression vector

An EcoRI-HincII DNA fragment coding for the carboxy terminal region of the FBD was isolated from plasmid pFN935-12 and ligated to plasmid pTV 194-80 digested with EcoRI and SmaI as described in Figure 6. The plasmid obtained was designated pFN 946-12.

3. Syntheses and cloning of DNA corresponding to nucleotides 468-599 of FN

Three pairs of chemically synthesized nucleotides were ligated to an EcoRI-DdeI FN fragment isolated from plasmid pFN932-18 (Figure 3) in the presence of pUC19 vector DNA (purchased from GIBCO BRL Co.) digested with EcoRI and XbaI as described in detail in Figure 7. The plasmid obtained was designated pFN 948-4.

4. Construction of a plasmid encoding the complete FBD region

In order to construct a plasmid which codes for the entire FBD, amino acid 1 to amino acid 262, an EcoRI-XbaI DNA fragment coding for FN was isolated from plasmid pFN948-4 and inserted into plasmid pFN 946-12 digested with EcoRI and XbaI as described in Figure 8. The plasmid obtained was designated pFN-957. This plasmid contains the complete coding sequence for FBD but does not express the FBD polypeptide as it lacks a ribosomal binding site (RBS).

5. Expression of the FBD under λ P_L promoter and cII RBS

An NdeI-HindIII fragment containing the FBD coding region and the T_1T_2 transcription terminators was isolated from plasmid pFN-957 and inserted into plasmid pTV 301 (Figure 33) digested with NdeI and HindIII as described in Figure 9. The resulting plasmid, designated as pFN 962-3, directs the expression of a FBD polypeptide under the control of λ P_L promoter and cII ribosomal binding site. Escherichia coli strains A1645 and A4255 transformed with this plasmid expressed only small amounts of the FBD polypeptide. The expression of the FBD polypeptide was detectable only by Western blot analysis using polyclonal antibodies directed against human plasma derived FN.

6. Expression of an FBD polypeptide under the λ P_L promoter and the β-lactamase promoter and ribosomal binding site

As the level of expression of the FBD polypeptide obtained with plasmid pFN 962-3 was low, we added a DNA fragment coding for the β-lactamase promoter and β-lactamase RBS (PBLA). The DNA fragment coding for PBLA was isolated from plasmid pBLA11 (ATCC Accession No. 39788) and inserted into plasmid pFN 962-3 digested with NdeI, filled in with Klenow enzyme and digested with EcoRI as described in Figure 10. The plasmid obtained, designated pFN 975-25, was deposited with the American Type Culture Collection under ATCC Accession No. 67832. This plasmid was used to transform Escherichia coli prototroph A4255 (F+).

These Escherichia coli cells were found to express the "complete" FBD polypeptide at levels comprising about 5-8% of the total cellular proteins. The polypeptide migrated on SDS-PAGE gels with an apparent molecular weight of 31 kD, hence it is referred to as the 31 kD polypeptide or the r31 kD FBD.

C. Fermentation and growth conditions

The clone expressing the r31 kD FBD polypeptide was fermented in rich medium (yeast extract and casein hydrolysate) containing ampicillin. Growth was carried out at 30°C. Expression was obtained upon induction at 42°C for 2 hours, and subsequently bacterial cell cake containing the r31 kD FBD polypeptide was obtained. Similarly, the clone expressing the r20 kD FBD was fermented and bacterial cell cake containing the r20 kD FBD polypeptide was obtained.

D. Refolding and purification of recombinant fibrin binding domain (r31 kD) polypeptide

The process is made up of three stages:

1. Crude processing of the bacterial cake.
2. Refolding/reoxidation.
3. Purification.

1. Crude processing

The cake is disrupted first in 5 volumes of 50 mM Tris-HCl/50 mM Na-EDTA, pH 8 (Buffer 1); the pellet is then treated with 1.2 volumes of Buffer 1 containing 100 mg/liter lysozyme (2 hours agitation at 37°C). Triton X 100 is added to the resulting suspension (to 1%), and

after 30 min. at room temperatur the suspension is centrifuged and the pellet is resuspended and washed twice with water. All these steps are performed by disruption of the pellet and centrifugation and the 31 kD stays in the pellet, as evidenced from SDS-PAGE gels.

The washed pellet is suspended in 14 volumes of 10 mM Tris-HCl/5 mM EDTA/2 mM PMSF/2mM 6-aminocaproate, pH 7.5 (Buffer A) and then treated successively with Buffer A containing: 1% decyl sulfate, 1% decyl sulfate/5% glycerol and 5% glycerol. The final treatment is with Buffer A without additives.

2. Refolding/reoxidation

Principle: To dissolve the pellet in 6M guanidine-HCl - GuCl - in the presence of a thiol reducing agent, such as glutathione - GSH - and to refold/reoxidize at a lower GuCl concentration by the addition of oxidized glutathione-GSSG.

The washed pellet from step 1 above is dissolved in 150-700 volumes of 6M GuCl/3mM GSH in Buffer A. The concentration of GuCl is lowered gradually, i.e., first 2 M, then 1 M and 0.5 M, while keeping the concentration of all other components constant, except for the volume, which at this stage is brought to 500-1000 fold higher than that of the pellet. At one of the intermediate concentrations of GuCl, i.e., between 0.5 and 2 M, refolding is initiated by the addition of 0.3 mM of GSSG and incubation at room temperature for 24-48 hours. The refolded 31 kD is then dialyzed against Buffer A without additiv s.

3. <u>Purification</u>

Concentration: The large volume of refolded 31 kD is first centrifuged to remove the insoluble pellet that contains no 31 kD and is then dialyzed against Tris-HCl, pH 7.8, before being concentrated and initially purified on a Heparin-Sepharose column.

EXAMPLE 3

Bacterial Binding Activity

Experiments have been performed on the binding of r31 kD FBD to bacterial suspensions of Staphylococcus aureus. Identical binding curves were obtained for radio-iodinated intact plasma fibronectin, the proteolytic 31 kD amino terminal fragment (p31 kD) derived from human plasma fibronectin, and r31 kD FBD.

The inhibition of bacterial binding by [^{125}I] p31 kD indicated that the recombinant 31 kD FBD competes with the authentic proteolytic fragment.

The bacterial binding activity of the r31 kD FBD is described in more detail in Example 7, Section II: Bacterial Binding Activity.

EXAMPLE 4

Inhibition of Bacteria Adhesion

To estimate the capacity of r31 kD FBD to interfere with the adherence of bacteria to the extracellular matrix in wounds, a competition assay was developed. In this assay, adherence of *Staphylococcus aureus* to a plastic surface coated with fibronectin and the interference of FBD with adherence were measured. Both authentic FBD and r31 kD FBD were active in inhibiting bacterial adhesion to the fibronectin coated surface.

EXAMPLE 5

Refolding and Purification of Recombinant 31 kD Fibrin-Binding Polypeptide of Fibronectin

The following is an improved procedure for the purification of the recombinant 31 kD fibrin-binding domain polypeptide (r31 kD) produced as described in Example 2.

The process is made up of three stages:

1. Crude processing of the bacterial cake.
2. Refolding/reoxidation.
3. Purification.

1. Crude processing

1.1 Washing of the pellet: The bacterial cell cake is disrupted first in 5 volumes of 50 mM Tris-HCl/50 mM Na-EDTA, pH 8 (Buffer 1). The pellet is then successively treated with Buffer 1 containing 100 mg/liter lysozyme (2 hours at 37°C), Buffer 1 containing 1% Triton X-100 (30 minutes at room temperature) and twice with water. All these steps are performed by disruption of the pellet and centrifugation; the r31 kD stays in the pellet, as evidenced from SDS-PAGE gels.

1.2 Extraction of the pellet: The washed pellet is suspended in 14 volumes of 10 mM Tris-HCl/5 mM EDTA/2 mM PMSF/2 mM epsilon-aminocaproate (Buffer A) pH 7.5, and then treated successively with Buffer A containing: (a) 1% decyl sulfate; (b) 1% decyl sulfate/5% glycerol; and (c) 5% glycerol.

The final treatment is with Buffer A without additives.

2. Solubilization and Refolding/reoxidation

A refolding/reoxidation procedure for the recombinant 31 kD polypeptide (r31 kD) has been developed and refined.

2.1 Principle: To dissolve the pellet in 6 M guanidine-HCl (GuCl) in the presence of a thiol reducing agent, such as glutathione (GSH) and to refold/reoxidize at a lower GuCl concentration by the addition of oxidized glutathione (GSSG).

2.2 Procedure: The extracted pellet is dissolved in 100-700 volumes of 6 M GuCl/3 mM GSH in Buffer A, pH 8.0. The concentration of GuCl in the dialysis buffer is lowered gradually, e.g., first 3 M, then 1.5 M and finally 0.5 M, while keeping the concentration of all other components constant. At one of the intermediate concentrations of GuCl, i.e., between 2 M and 1 M, refolding is initiated by the addition of 0.3 mM of GSSG and incubation at pH 8 at room temperature for 48-72 hours. The refolded r31 kD is then dialyzed against Buffer A at pH 8.5, without additives.

Example: Approximately 10 grams of extracted pellet (see 1.2) were homogenized and dissolved in 1 liter of Buffer A/6 M GuCl/3 mM GSH/pH 8 and the suspension was stirred for 14 hours until it was a clear solution. This solution was dialyzed for 24 hours against four liters f Buffer A which additionally contained 3 mM GSH and 3 M GuCl, pH 8. Subsequently, the resulting solution was dialyzed

for 24 hours against 8 lit rs of Buffer A containing 3 mM GSH, pH 8. The resulting solution was dialyzed twice against 10 liters of Buffer A containing 0.3 mM GSH and 0.3 mM GSSG, pH 8. The process of dialysis, during which reoxidation also occurred, lasted approximately 80 hours. Finally, the glutathione was removed from the refolded protein by dialysis against 10 liters of Buffer A, pH 8.3 - 8.5. This step was performed twice. Subsequently, the solution was loaded on a phenyl-Sepharose column.

2.3 Alternative procedure: Similar results have been obtained when cysteine (3 mM) was used instead of glutathione and cystine (0.3 mM) instead of oxidized glutathione.

2.4 Use of thioredoxin: Attempts were also made to increase the rate of reoxidation of the r31 kD, by using thioredoxin. BAsed on SDS-PAGE profiles run in the absence of mercaptoethanol (ME), thioredoxin reduction and reoxidation of "scrambled" material seems to yield a more homogeneous preparation of r31 kD, but the concentration of thioredoxin which had to be used was about 100 µM. "Scrambled material" is r31 kD polypeptide which is apparently improperly folded due to the formation of one or more incorrect disulfide bonds.

3. Purification

3.1 Phenyl-Sepharose chromatography: The large volume of r folded r31 kD is first centrifuged to rem v the insoluble pellet which contains either "scrambled" r31 kD or contaminants. The supernatant is

brought to 0.2 M ammonium sulfate in Buffer A and loaded onto a phenyl-Sepharose column equilibrated with Buffer A containing the same ammonium sulfate concentration. The r31 kD polypeptide is then purified by lowering the salt concentration, i.e., by elution in Buffer A.

Example: After reoxidation of the crude protein mixture, extracted from a 10 gram pellet, the suspension of refolded and "scrambled" r31 kD, as well as insoluble contaminants, is subjected to centrifugation at 13,000 rpm (17,000 x g) in a high-speed Beckman centrifuge equipped with a J-14 rotor. The supernatant (1,280 ml) was brought to 0.2 M in ammonium sulfate (AS) and loaded onto a 45 ml column of phenyl-Sepharose previously equilibrated with Buffer A containing 0.2 M AS. The column was washed with 150 ml of the same solution, followed by 150 ml of Buffer A, 50 ml of water and 50 ml of 6 M GuCl (Figures 11 and 12).

3.2 Heparin-Sepharose and ion-exchange chromatographies: The final step of purification of the r31 kD polypeptide is chromatography on Q-Sepharose from which it elutes in the flow-through fraction or on Heparin-Sepharose from which it is eluted by using a salt gradient. It also binds to S-Sepharose, but the eluted material is still contaminated with most of the impurities.

Example: Approximately 1/2 of the Buffer A peak was concentrated and purified on a 10 ml Heparin-Sepharose column, from which it was eluted by a solution of 0.5 M NaCl in Buff r A (Figure 13). Th concentrated 31 kD was dialyzed against Buffer

A, pH 8.5, before being loaded on a 40 ml column of Q-Sepharose, which had previously been equilibrated with the same buffer. The purified r31 kD polypeptides, which eluted in the flow-through and wash fractions were concentrated by lyophilization, before being characterized. The column was washed free of the contaminant proteins by a step of 1 M NaCl (Figure 14). The purified material is greater than 95% pure (Figure 11).

3.3 <u>Re-extraction of the pellet</u>: After the refolding procedure the pellet was re-extracted and treated as above, since it still contained more than 50% of the r31 kD polypeptide probably in "scrambled" form. The total yield (including the re-extraction step) of the process, after 3 columns, was about 10% (Table A).

3.4 <u>Characterization</u>: The r31 kD polypeptide has been characterized and compared to its plasma derived counterpart in terms of its purity (purity profile on reduced gels of SDS-PAGE), migration position in non-reduced gels of SDS-PAGE (Figure 11), apparent molecular weight (approximately 37 kD) on Superose 12 (Figure 15), immunoblot and behavior on Heparin-Sepharose (the NaCl concentration for elution of both materials from Heparin-Sepharose was found to be approximately 0.32 M). In all of these assays the r31 kD polypeptide is similar to plasma derived fibrin binding domain.

4. Comparison between various forms of the r31 kD in terms of their reoxidation-refolding

Three different forms of the r31 kD polypeptide have been defined:

Form a: The polypeptide as it is obtained from the washed pellet, after dissolution in 6 M GuCl, i.e., in "scrambled" form.

Form b: The fully reduced polypeptide, present after treatment with a reducing agent such as GSH in the presence of 6 M GuCl.

Form c: The reoxidized-refolded polypeptide, obtained by treatment with the GSH/GSSG as described above.

These three forms can be distinguished from one another by:

(i) Their migration on SDS-PAGE gels in the absence of reducing reagents (i.e., without ME), and in the presence of the thiol-trapping agent iodoacetamide (Figure 11); and

(ii) Their reaction with anti-plasmatic 31 kD on immuno-blots of gels run in the absence of ME. Only the correctly reoxidized-refolded form reacted with the antibody.

The work described above on reoxidation in the presence of GSH/GSSG (or in the pr s nce of cysteine/cystine) has shown that the r31 kD polypeptid ref lds with time to

f rm (c) which is indistinguishable from that of the plasma derived fibrin binding domain.

Characterization of the "scrambled" r31 kD polypeptide: The polypeptide seems to be less soluble in this form, as evidenced from the large amounts remaining in the pellet after extraction.

Also the scrambled polypeptide differs from the refolded polypeptide in its binding characteristics to both phenyl-Sepharose and Q-Sepharose.

Characterization of the fully reduced r31 kD polypeptide: This polypeptide form is both more soluble and more homogeneous than the "scrambled" one. After solubilization in 6 M GuCl in the presence of GSH (or DTT or cysteine), and then lowering the concentration of denaturant to almost zero, the "reduced" polypeptide can be purified on phenyl-Sepharose in the presence of GSH. Finally, the polypeptide is "reoxidized" with GSSG at 1/10 of the concentration of GSH, i.e., 0.3 mM. This did not yield refolded 31 kD, but a form of "scrambled" polypeptide different from that described in the previous paragraph, probably because the reduced polypeptide slowly autooxidizes to a scrambled form, even before it is exposed to GSSG.

5. Preparation of reduced-carboxamidated 31 kD polypeptide

Purified plasma derived or recombinant 31 kD polypeptide (approximately 0.6 mg/ml) were reduced in 4.3 M Guanidinium Hydrochloride (GuCl), 40 mM ß-mercaptoethanol (ME), in 10 mM Tris-HCl, pH 8.5 for 24 hours at r m temperature.

Carboxamidati n was achi ved by adding iodoacetamide to the polypeptide in four-fold excess over the concentration of ME and the solution incubated for 1 hour at room temperature. Subsequently the GuCl concentration was reduced by gradual dialysis (to 3M, 2M, 1M and 0.5M GuCl), before being dialyzed against Buffer A. The precipitate formed was centrifuged and the concentration of the resulting 31 kD polypeptide in the supernatants were 0.34 and 0.19 mg/ml for the plasma derived and recombinant 31 kD, respectively. The reduced carboxyamidated polypeptide is also termed a reduced/blocked polypeptide because the S-H groups are blocked to prevent reoxidation.

Table A

Purification of r31kD

STEP	VOLUME (ml)	PROTEIN CONC. (mg/ml)	TOTAL PROTEIN (mg)	PURITY (%)	AMOUNT 31kD (mg)	YIELD (%)	PURIF. DEGREE
Extracted pellet			~2000	35	~700	100	1
Refolding	1280	0.332	425	70	295	42	2.0
Phenyl-S.	220	0.56	123	85	105	15	2.4
*(1/2)			61		52		
Heparin-S.	16	2.19	35	90	32	9	2.6
Q-Sepharose	100		23a	>95	22	6	>2.7
Re-extracted pellet			234			23	
Phenyl-S.			96			14	

§ Purity determined from SDS-PAGE gels (=HE)

a Measured after lyophilisation.

EXAMPLE 6

Pharmacodynamics of the r31 kD, r20 kD and r12 kD Fibrin Binding Domain Polypeptides

The intensity and resolution of a clot (thrombus) image is governed by the interplay of the rate of incorporation of the radiopharmaceutical and its blood clearance rate. In order to elucidate the metabolic behavior of the r31 kD fibrin-binding domain, and to compare it to fibronectin (FN), the r31 kD fibrin binding domain and plasma fibronectin were both iodinated with ^{125}I by the ICl method (24) and injected intravenously into rats. The results are shown in Figure 16 which represents the pharmacokinetic behavior of ^{125}I-r31 kD FBD and ^{125}I-FN. Blood samples were withdrawn at the times shown on the graph.

Figure 16 demonstrates that the clearance rates of the two radioactive molecules are different and after 5 hours, only 3% of the r31 kD FBD but 20% of FN respectively remain in circulation.

Some of the rats were kept in individual metabolic cages, and accumulated urine and feces were collected at 7 hours and 24 hours. About 30% of the injected ^{125}I-r31 kD radioactivity was excreted in the urine during the first 7 hours, and more than 90% was excreted after 24 hours. All of the urinary radioactivity was trichloroacetic acid-soluble, which is indicative of proteolytic degradation. The analysis of a variety of organs (kidney, stomach, liver, lung, uterus, ovary, adrenal, colon, ileum, skin, brain, eye, muscle, bladd r, heart, spleen, trachea, aorta and vena-cava) did not reveal any specific accumulation, and the kinetics of disappearance of the

radioactivity followed a pattern similar to that of the blood. In most of the organs, the specific radioactivity (cpm/gram tissue) was lower than that of the serum.

The results indicate that exogenous recombinant 31 kD amino-terminal polypeptide of FN is moderately degraded and excreted in the body. The pharmacokinetic behavior is not consistent with a first-order kinetics, which may indicate that the polypeptide is moderately distributed in the tissues and body compartments other than blood. This is also evident from the finding that the degree of degradation does not increase during the 4-24 hour period, thus reflecting a gradual release of the polypeptide from body compartments. The exclusive and relatively early appearance of the metabolites in the urine indicates that the polypeptide is readily excreted through the kidneys. The lack of accumulation of the material in the liver may be an indication that this organ is not a major locus of degradation and is not involved in detoxification.

The relatively short half-life of r31 kD FBD is important for its possible use in diagnostic imaging of thrombi. The recombinant 31 kD FBD (r31 kD) may be labeled radioactively or by other means and then introduced into the blood for the purpose of imaging thrombi.

The shorter half-life of the molecule is also important when utilizing it to prevent clot formation. By contrast, heparin, the current therapeutic agent of choice, suffers from a very long half-life.

A similar experiment was performed using iodinated 31 kD fibrin binding domain of plasmatic fibronectin and

similar pharmacokin tics and distribution of radioactivity were observed.

The 31 kD polypeptide was obtained by cleavage of plasmatic FN as follows: the plasmatic FN was purified on a Gelatin-Sepharose column from which it was eluted and stored in 1 M guanidinium hydrochloride. Thereafter, 206 mg of FN, after dialysis against 10 mM of Tris-HCl, were digested with 0.01% of TPCK-trypsin at 37°C for 5 minutes. The tryptic digest was loaded on a DE52 column (6 ml) and 1/5 of the flow-through fraction (50 ml) was applied to a CM-Sepharose column (3 ml) and eluted with a NaCl gradient (0-0.5 M). About 80% of the polypeptide was recovered in the salt gradient (peak at about 220 mM) and after dialysis to remove the salt about 1/2 of the polypeptide was loaded on a Heparin-Sepharose column (1.5 ml) and eluted with 0.5 M NaCl. Approximately 75% of the polypeptide was recovered in this fraction, i.e., about 1 mg (about 40% of the theoretical yield). This fraction was >90% pure 31 kD polypeptide, and was iodinated by the method described above.

Note that plasmatic 31 kD FBD contains the first 259 amino acids of FN, whereas the recombinant 31 kD FBD contains the first 262 amino acids of FN.

<u>Pharmacokinetics of the r20 kD and r12 kD fibrin binding domain polypeptides</u>

Similar experiments were performed using labeled r20 kD and r12 kD fibrin binding domain polypeptides produced as described in Examples 2, 9 and 10. The pharmacokinetics of these p lypeptides was found to be very similar to that of th r31 kD polypeptide.

EXAMPLE 7

Biological activity of the recombinant Fibrin Binding Domain (r31 kD)

The biological activity of the purified recombinant 31 kD FBD polypeptide was compared to biological activity of either human plasma derived FN or a 31 kD FBD polypeptide derived from partial tryptic digest (Example 6) of human plasma derived FN. The biological activities assayed were binding to fibrin clot *in vivo* and *in vitro*, binding to bacteria (*Staphylococcus aureus*) and binding to extracellular matrix.

I. Fibrin binding activity

Two sets of experiments were carried out. In the first set, the binding of ^{125}I-r31 kD to fibrin was monitored during clot formation (Reaction I), while in the second set of experiments, the binding of ^{125}I-r31 kD to fibrin was monitored at various time periods after clot formation (Reaction II). Thrombin or Ca^{++} were added to the mixtures in order to enable clot formation in citrated blood.

Materials and Methods

Fibronectin Fibrin Binding Domain: FBD

A. Binding of FBD to fibrin:

Plasma derived 31 kD FBD was derived by partial tryptic digest of human fibronectin (see Example 6).

Production of r31 kD FBD in Escherichia coli and its subsequent purification from the pellet of cell lysate was performed according to Example 5. Fibronectin was obtained from human plasma. ^{125}I-labeling of FBD and FN was carried out by the ICl method (24). The labeled polypeptides having specific activity of 20-200 cpm/ng, were stored at -20°C in small aliquots in a solution of 0.1% BSA-PBS and used within 2 weeks.

1. Binding of ^{125}I-FBD to fibrin clot during its formation (Reaction I):

The complete reaction mixture in siliconized microfuge tubes contained in a final volume of 250 µl the following components:

20-200 µl human whole blood (fresh, non-citrated, or 1-7 days old, citrated, as indicated in the figure legends)

0.1% BSA
5 mM $CaCl_2$
1U/ml Thrombin
^{125}I-r31 kD FBD

When binding was measured using non-citrated blood, $CaCl_2$ and thrombin was not added ("Naive Thrombii").

All ingredients were prepared in PBS. The reaction was incubated at 37°C for 30 minutes. The reaction was terminated by the addition of EDTA (25 mM), and centrifugation in a microfuge centrifuge at maximum speed for 3 minutes. The supernatant was discarded and the pellet was wash d twic in 1 ml PBS, 0.1% BSA, 5 mM EDTA, 1 mM

PMSF. The radioactivity in the pellet was monitored by a gamma counter.

When competition with non-radioactive polypeptide was carried out, the competing polypeptide was added together with the ^{125}I-labeled polypeptide at the concentrations indicated in the figure legends.

2. Binding of ^{125}I-r31 kD FBD to preformed fibrin clots (Reaction II):

The reaction mixture contains the same components as indicated for Reaction I, except for the ^{125}I-r31 kD. The first incubation was carried out at 37°C for 30 minutes, and only then the ^{125}I-r31 kD was added and the reaction was further incubated for a second period of 30 minutes. Reaction II was terminated and measured as described above for Reaction I.

Results

A. Binding of ^{125}I-r31 kD to fibrin: effect of thrombin and Ca^{++}

The effect of thrombin and Ca^{++} on ^{125}I-r31 kD binding to fibrin during clot formation (Reaction I) or to preformed clots (Reaction II) was studied in citrated human whole blood (Figure 17).

Hirudin, a specific inhibitor of thrombin (25) reduced the binding of ^{125}I-r31 kD to the fibrin clot in Reaction I, indicating that thrombin is needed for the binding. When thrombin is inhibited there is a reduction in clot formation and l ss fibrin is available for binding. Th addition of citrate to blood significantly r duces the

concentration of free Ca^{++} in the serum and therefore the addition of Ca^{++} for fibrin clot formation is obligatory. However, binding of r31 kD to a preformed clot is reduced when carried out in serum which has been already depleted of free Ca^{++} ions by the preformed clot. Addition of Ca^{++} to the Ca^{++} depleted serum increases the r31 kD binding. This effect of Ca^{++} on binding was dramatically demonstrated when binding was measured in PBS. The addition of Ca^{++} increased the binding, even to a higher extent than observed in reaction I. Thus, both clot formation and r31 kD binding are Ca^{++} ion dependent.

Thus, binding of ^{125}I-r31 kD to fibrin in Reaction II increases when the assay is carried out in PBS (Phosphate Buffer Saline solution) instead of serum.

B. Release of ^{125}I-r31 kD from fibrin clot by Plasmin

In order to determine whether the ^{125}I-r31 kD is covalently bound to the fibrin in the clot, plasmin, which is known (26,27) to cleave the N-terminal domain of plasma derived FN between amino acids Arg^{259} and Thr^{260} was added to the clot after ^{125}I-r31 kD was bound. The incubation with plasmin was carried out for various time intervals (Figure 18).

The results demonstrate that plasmin caused a reduction in the radioactivity bound to the clot (pellet). Reduction of radioactivity in the pellet was time dependent and could be attributed to the fact that plasmin cleaved the r31 kD FBD. The amount of radioactivity monitored in the supernatant increased with time. Upon loading f the plasmin soluble reaction fraction (the supernatant) on SDS polyacrylamid gels it is possible to detect the shortened form f the cl av d ^{125}I-

r31 kD. The release of ^{125}I-r31 kD from the fibrin clot occurs only by plasmin cleavage and not by heating with a solution containing SDS, EDTA and ß-mercaptoethanol to 100°C, thus demonstrating that ^{125}I-r31 kD is covalently bound to the fibrin clot.

3. Binding of ^{125}I-r31 kD to fibrin during clot formation: effect of unlabeled r31 kD and other molecules

The ability of various cold recombinant and plasma derived FBD preparations as well as human plasma derived fibronectins at different concentrations to interfere with the binding of ^{125}I-rFBD to fibrin during clot formation (Reaction I) was studied (Figure 19). The results obtained demonstrate that the binding of 0.15 µM ^{125}I-rFBD to fibrin was similar or even increased in the presence of unlabeled r31 kD, unlabeled plasma derived 31 kD or unlabeled FN at concentrations up to 20 folds excess (3 µM).

However, when the amount of newly formed fibrin clot was reduced, using either suboptimal concentrations of $CaCl_2$ and thrombin (1 mM and 0.3 units/ml, respectively), or by reducing the volume of the blood in the reaction to 1/10 of the amount originally specified, the competition between the unlabeled FBD and the binding of ^{125}I-FBD for binding to the clot became significant.

In order to further study the specificity of the FBD binding reaction, we compared the binding of r31 kD to the binding of its reduced forms (Example 5).

For these studies w used n batch f r duced-carboxamidated plasma derived 31 kD, on batch f reduced-

carboxamidated r31 kD and one batch of fully-reduced r31 kD all prepared as described in Example 5. Surprisingly, these various reduced forms of 31 kD FBD caused a dramatic reduction in the binding of the ^{125}I-FBD (reduction of 40-80% by 0.3-3.0 μM; Figure 19). We have also noticed a dramatic decrease in the size of the newly formed clot (Reaction I) in the presence of the fully reduced FBD. Clot formation was totally inhibited in the presence of high concentrations of the fully reduced FBD (above 5 μM).

Since a similar inhibitory effect was exhibited by the various reduced forms of the 31 kD proteins on the binding of refolded ^{125}I-FBD to a preformed fibrin clot (Reaction II), the possibility of interference with the crosslinking reaction of the FBD to the fibrin clot catalyzed by the serum transglutaminase factor XIIIa, was suggested.

4. Binding of ^{125}I-FBD to the fibrin thrombi (Reaction II): effect of transglutaminase inhibitors

Transglutaminases are a class of calcium ion-dependent enzymes that catalyze an amidation reaction in which a carboxamide group of peptide bound glutaminyl residues and primary amines, including the epsilon amino group of peptide bound lysyl residues, are crosslinked. Plasma FN is a substrate for transglutaminase from plasma, factor XIIIa, (thrombin activated blood coagulation factor XIII) or liver; FN can be crosslinked to itself, fibrin and collagen.

The glutaminyl residues which are susceptible to factor XIII crosslinking are localized in the FBD region of FN (28).

The binding of ^{125}I-FBD to preform d fibrin clot (Reaction II) in the presence of various concentrations of the primary amines spermidine and putrescine, the classical transglutaminase inhibitors, was studied (Figure 20). The reaction was 50% inhibited by about 5 mM spermidine or putrescine. In parallel to the expected inhibition by the primary amines, a dramatic inhibition of the binding was also observed by the reduced-carboxamidated FBD, with a half maximal reduction at around 2.5 μM.

5. Binding of ^{125}I-r31 kD to preformed fibrin clot; effect of aging on binding

For imaging purposes, it was important to determine the effect of clot aging on FBD binding capacity.

The binding of ^{125}I-r31 kD to preformed fibrin clot was determined. ^{125}I-r31 kD was added to the preformed clots (1, 4 or 24 hours) and binding to fibrin was monitored (Figure 21).

The binding of ^{125}I-r31 kD to the fibrin clot was higher after 24 hours than the binding after 1 or 4 hours, presumably due to the fact that the clot formed became larger with time.

As indicated in experiment I of this example, the presence of free Ca^{++} ions in citrated blood is important in order to obtain optimal binding and crosslinking of ^{125}I-r31 kD to the fibrin clot. Thus, in serum where Ca^{++} was already depleted by the preformed clot the binding of ^{125}I-r31 kD to the fibrin clot was low while in the PBS c ntaining 5 mM $CaCl_2$, the binding was high at all incubation times.

When 300 μg/ml f plasma derived FN was added as competitor in the presence of Ca^{++}, there was a 20% reduction in ^{125}I-r31 kD binding to the fibrin clot probably due to some competition on the available fibrin binding sites.

6. Binding of ^{125}I-r31 kD to "Naive" thrombi (Reactions I and II): effect of thrombi age on the binding

The ability to differentiate between "old" (preformed) and "newly formed" thrombi, is an important requirement of a probe for thrombus imaging. Figure 22 shows experiments designed to compare ^{125}I-r31 kD binding to "old" and "newly formed" clots. In the first experiment ^{125}I-r31 kD was added at the same time as the initiation of clot formation ("newly formed") and was allowed to interact with the clot during seven days of incubation. It is evident that FBD is incorporated efficiently and the amount incorporated increased during the first two days, probably representing an increase in the clot size during that period. In the second experiment ^{125}I-r31 kD was added to aged clots (1-7 days old) for a limited period of 2 hours ("preformed"). The extent of FBD incorporation remained constant, regardless of the clot age. Note, however, that binding in this protocol is lower than in that of the first experiment. These experiments suggest that the probe ^{125}I-r31 kD may be used in two different protocols to differentiate between "old" clots - thrombi and those which are still in the process of growing.

7. Plasma "Thrombin Time" ("TT")

The effect of the r31 kD polypeptide on clotting of whole blood was measured using the clinical laboratory parameter defined as Thrombin-Time. In this reaction aliquots

of 100 µl citrated healthy human plasma were mixed with 100 µl PBS and either the transglutaminase inhibitor spermidine, or with reduced-carboxyamidated p31 kD or reoxidized-refolded r31 kD. 200 µl thrombin solution was added to each aliquot while continuously mixing. The time from the addition of the thrombin to the formation of clot was measured, and is expressed as "TT" in seconds (Table B).

8. Binding of ^{125}I-r31 kD to fibrin (Reaction I): effect of exogenous transglutaminase and "reduced-carboxamidated" FBD

In Section 4 of this example we demonstrated that transglutaminase inhibitors reduce the binding of ^{125}I-r31 kD to clots. In this section we studied the effects of exogenous transglutaminase from pig liver on the binding reaction.

The results presented in Figure 23 demonstrate that the addition of the exogenous transglutaminase dramatically increases the binding of ^{125}I-r31 kD to clots, indicating that this enzyme may be a rate limiting step in this reaction.

Moreover, both endogenous and exogenous transglutaminase dependent binding activities were equally decreased by the reduced-carboxamidated FBD (approximately 56% and 72% inhibition by 0.3 µM and 1.5 µM "reduced" FBD, respectively).

These results strongly indicate that the "reduced" forms of FBD inhibit the binding of the refolded form of FBD to the fibrin cl t by interfering with th transglutami-

nation and cross-linking reaction. This probably causes destabilization of the fibrin clot.

9. Binding of ^{125}I-FBD to Extra Cellular Matrix (ECM)

Adhesive molecules such as von Willebrand factor, fibronectin, fibrinogen, thrombospondin, collagen and laminin bind to ECM formed by removal of endothelial cells. r31 kD FBD can serve for imaging the initial steps in plaque formation at the site of injury. The binding of ^{125}I-r31 kD at various concentrations to ECM was studied in the presence or absence of thrombin. The results demonstrated that the binding of ^{125}I-r31 kD at low concentrations to ECM was not affected by thrombin (0.3 μM). At higher concentrations of the thrombin binding of r31 kD was slightly higher indicating that the number of binding sites naturally present are limited and thrombin digestion might expose additional binding sites (Figure 24).

The fact that ^{125}I-r31 kD binds to ECM indicates that it will be useful for imaging the initial plaque formation in the denudated blood vessel.

Table B

THROMBIN TIME ("TT")

Effect of FBD ("reduced" and "folded") and Related Molecules

	Thrombin Conc. (µ/ml)	Additions	"TT" Values (Seconds)	(%)	(\)
I	2.5	-	25.5	100	-
		1mM Spermidin	44	172	72
		1uM "R"FBD	34	133	33
		2.5 µM "R"FBD	39	152	52
		2.5 µM "F"FBD	26.5	103	03
II	1.25	-	55.4	100	-
		1mM Spermidine	114	205	105
		2.5µM "R"FBD	81	146	46
		2.5µM "F"FBD	58	104	04

"R" FBD = Reduced-carboxamidated p31kD.

"F" FBD = Refolded r31kD.

II. Bacterial Binding Activity

The involvement of fibronectin in adhesion to, and invasion of, wounds by a wide range of gram-positive bacteria is well established (18). The fibrin binding domain of authentic plasma derived FN has been shown to interact with high affinity to specific receptors on the surface of bacteria. The sites at which Staphylococcus aureus typically initiates infection are rich in FN, e.g. blood clots and subendothelium. Furthermore, exogenous FN enhances bacterial adhesion to these sites. FN binds to S. aureus through saturable, specific surface protein receptors. Scatchard analysis has revealed high affinity receptors with binding constant of 5×10^{-9} M, and a range of 100-20,000 receptors per bacterium (19). The expression of FN receptors correlates with invasiveness and pathogenicity of the clinical isolates. Removal of the receptors from S. aureus by mechanical means, or by growth of the bacteria in the presence of antibiotics decreases their ability to adhere to FN. As FN is a divalent molecule consisting of multiple functional domains with cell binding and collagen binding activities in addition to bacterial binding, it can anchor the bacteria to the wound via the various components of the extracellular matrix as well as via the FN receptor in tissue cells.

Materials and Methods

Binding of Bacteria to labeled FN or FBD in solution

A. Direct binding reaction

Various concentrations of ^{125}I-r31 kD FBD or ^{125}I-FN, were added to 5 x 10^8 S. aureus bacteria in a PBS solution additionally containing 0.1% Tween and 1% BSA. The final volume was 1 ml. Total radioactivity in the reaction was assayed using a 20 µl aliquot taken immediately after the addition of the bacteria.

The mixture was incubated for 2 hours at 20°C.

The amount of binding was assayed by removing 100 µl of the incubation mixture and layering on top of 0.5 ml PBS layered on 3 ml 10% percol, 0.15 M NaCl in a 5 ml siliconized tube. This was then centrifuged at 1,350 x g (4,000 rpm in SW bucket rotor) for 15 minutes at 20°C. The supernatant was aspirated and the pellet assayed for radioactivity.

B. Competition with unlabeled FN, FBD and related molecules

The procedure followed was identical to the above procedure except that 3 µg/ml ^{125}I-p31 kD was used and the specified amounts of the competing molecule (FN or FBD) was also added to the initial binding mixture.

II. Binding of radioactively labeled bacteria to immobilized FN

Plastic vials were coated with 0.3 ml of 50 µg/ml FN, or 1% BSA.

The tubes were incubated with shaking at 4°C overnight. The tubes were then washed with 5 ml PBS three times. Then 0.3 ml of 1% BSA in PBS was added and the tubes were further incubated with shaking for 2-3 hours at 20°C (for blocking free sites).

In indirect-binding experiments, the bacteria were pre-incubated with inhibitor, at 4°C for 2 hours.

The bacteria (4 x 10^6 pfu/ml, 3 pfu/cpm) were added to the vials at concentrations indicated in the figure legends. The final volume of the assay mix was 0.3 ml PBS. The mix was slowly agitated at 4°C for 90-120 minutes.

The tubes were then decanted and washed with 5 ml PBS three times.

5 ml of scintillation-liquid was added when assaying for binding of 3H-labeled bacteria.

III. Binding of labeled bacteria to catheters

Catheters ["UNO" sterile bronchial plastic catheters (18 CH size; Unoplast A/S, Denmark)], were cut to 1 cm and 2 cm pieces, weighed and then cut once lengthwise.

The catheter pieces were incubated with 50 μg/ml FN at 4°C overnight with shaking. The controls were incubated with PBS under the same conditions.

The FN solution was then decanted and the catheters were washed three times in PBS. BSA-blocking was performed by adding 1% BSA in PBS for 1-2 hours at 20°C. The catheters were again washed three times with PBS.

Bacterial binding was performed as described in the previous section except that the catheter pieces were added to the assay mixture. The final volume used was 3 ml. The reaction was performed in 10 ml tubes. The reaction was incubated for 2 hours at 20°C. For the binding assay ^{125}I-S. aureus (4×10^6 pfu/ml, 3 pfu/cpm) were used. (When H3-leucine-labeled S. aureus was used the specific activity was 1 cpm/3.3×10^3 pfu. Labeling was performed according to P.B. Russel et al. (29). When ^{125}I-labeled S. aureus was used the specific activity was 1 cpm/3 pfu. Labeling was performed according to A.E. Bolton and W.M. Hunter (30).) The competing molecules at the designated concentrations were preincubated with the bacteria for 30 minutes at 20°C. The catheters were then washed three times with PBS and then directly counted in a gamma counter.

Results

A. Binding of bacteria to ^{125}I-FN or FBD in solution

I. Direct Binding

Experiments were performed in order to determine the binding f ^{125}I-FN r ^{125}I-rFBD t S. aureus bacteria in suspension. Various amounts of radioactive FN or r31 kD

were added to 5 x 10^8 bacteria incubated for 2 hours and then centrifuged over a 10% Percoll-saline solution. Radioactivity was monitored in the pellet (Figure 25).

The results showed increased binding of ^{125}I-rFBD (r31 kD) to the bacteria in suspension as compared to the binding of the ^{125}I-FN.

This increased binding of ^{125}I-rFBD to S. aureus as compared to ^{125}I-FN binding to S. aureus can be attributed to a higher affinity of a monovalent domain in comparison to bivalent multidomain of intact plasma derived FN.

II. Binding of ^{125}I-plasma derived 31 kD FBD (p31 kD) to S. aureus; competition with "native" unlabeled FN, FBD and related molecules

A fixed amount of ^{125}I-p31 kD (3 µg/ml) was incubated with 5 x 10^8 bacteria in the presence of increasing amounts of various FBD molecules as competitors (Figure 26).

The results demonstrate that "native" FN, p31 kD or rFBD inhibited the binding of ^{125}I-p31 kD to S. aureus in a similar fashion, indicating that rFBD is as active as the natural plasma derived molecules. However, the reduced forms of recombinant or plasma derived FBD only minimally inhibit the binding of ^{125}I-FBD to the bacteria, indicating that proper folding is necessary for binding. A related recombinant polypeptide (33 kD cell binding domain of FN) which does not have a bacterial binding site did not inhibit ^{125}I-pFBD binding to S. aureus.

B. Binding of labeled S. aureus to immobilized FN

To estimate the capacity of rFBD (r31 kD) to interfere with the adherence of bacteria to the extracellular matrix in wounds, a competition assay was developed. In this assay, adherence of S. aureus to plastic surface coated with FN, and the interference of FBD with the binding was measured (see Figure 27).

The results demonstrate that the adhesion of S. aureus to FN coated plastic vials was inhibited following pre-incubation of S. aureus with FN, pFBD or rFBD. The extent of inhibition by these molecules was similar. A non-related protein, BSA, which does not have S. aureus binding sites, did not cause any inhibition in adhesion of radioactive labeled S. aureus to FN coated plastic vials.

C. Binding of S. aureus to bronchial catheters: effect of FBD and heparin

Catheter sepsis due to various species of S. aureus contributes to the high incidences of serious clinical complications.

We have examined the ability of S. aureus to bind FN coated catheters. Figure 28 demonstrates that the binding of S. aureus to the FN coated catheters is quite high, approximately 10^4 PFU/cm^2.

Preincubation of bacteria with increasing concentrations of r31 kD reduced the binding of the bacteria to the catheters. The IC_{50} f r this inhibition is between 0.08

- 0.8 μM (Figur 28). Similar inhibition was also obtained with r20 kD FBD and p31 kD FBD.

Systemic administration of heparin and/or the use of heparin bonded polyurethane catheters is reported to decrease the incidence of thrombosis.

Therefore we also measured the inhibitory effect of FBD on S. aureus attachment to the catheters in the presence of 5 μM heparin. The results (Figure 28) demonstrate that heparin did not affect the binding of the bacteria to catheters, however, r-FBD inhibition of bacterial binding remains constant even in the presence of heparin. This indicates the utility of r31 kD for inhibition of S. aureus colonization and sepsis in a clinical setting, even in the presence of heparin.

Conclusion

These results demonstrate that the r31 kD FBD or the plasma derived 31 kD FBD may be used therapeutically in preventing bacterial colonization of wounds. The various FBD polypeptides will be formulated in suitable pharmaceutical formulations well-known to the average man of the art, and then used to "irrigate" or "flood" or treat the wound area for a suitable period of time, thereby preventing bacterial colonization of the wound.

EXAMPLE 8

Adhesion of plasma derived 31 kD FBD to thrombi in a rabbit aorta lesion model

In order to demonstrate the ability of the fibrin binding domain to adhere to thrombi *in vivo*, a model of thrombus formation in rabbits was used. In this model, the endothelial layer of a segment of the aortal wall is removed with a balloon catheter, thus exposing the lamina intima. This results in the subsequent formation of a film of thrombi in the lesion area. It has been demonstrated by Uehara et al. (1) that labeled FN given systemically to rabbits exhibited extensive binding to such thrombi, as indicated by a higher specific radioactivity in the lesion part of the aorta as compared to adjacent untreated segments.

In order to compare the plasma derived 31 kD FBD polypeptide with intact plasma-derived FN, the two molecules were iodinated with ^{125}I using the ICL method. Two hours after de-endothelialization, the radio-labeled molecules were injected intravenously into rabbits (20 μCi; 100 μg/kg; 3 rabbits per group). At 72 hours after injection, the aortas were removed, the scraped (abdominal) and intact (thoracic) areas were separated and each part was cut into several segments (5-6 segments for the lesion part and 2 segments for the control part). The tissue pieces were weighed and radioactivity counted.

Figure 29 summarizes the specific activity values found in the sequential aorta slices in rabbits injected with ^{125}I-FN (rabbits No. 1-3) or ^{125}I-p31 kD FBD (rabbits 4-6). As can be seen in Figure 29, enhanced localization of th labeled m lecules was found in th de-endothelialized

segments (thrombus zone). Also mor radioactivity was localized in the clots when labeled p31 kD FBD was used than when labeled FN was used.

These results demonstrate that molecules comprising the FBD moiety should be useful for the imaging of arterial thrombus clots.

When used for imaging it may be desirable to attach to the FBD (plasma derived or recombinant) other markers useful in imaging such as enzymes or radio-opaque inert molecules.

EXAMPLE 9

Expression and Fermentation of Additional Fibrin Binding Domain (FBD) Polypeptides

In Example 2 the expression of a partial r20 kD FBD and the full-length r31 kD FBD was described and in Example 24 an improved procedure for refolding and purification of the 31 kD FBD was disclosed. The construction of plasmids for expression of additional FBD polypeptides is now described.

A. Expression of r12 kD FBD polypeptide

Plasmid pFN 975-25 (Figure 10) expresses the full-length r31 kD FBD of fibronectin and from it plasmid pFN 196-2 which expresses a partial FBD was constructed as shown in Figure 36. This plasmid was transformed into Escherichia coli strain A1645 and thence into Escherichia coli strain A4255 and deposited in A4255 in the ATCC under Accession No. 68328. These transformed cells were found to be good expressors of the partial FBD polypeptide in amounts comprising about 5% of the total cellular protein. The polypeptide has a mobility of about 12 kD on reduced SDS polyacrylamide gels as determined from the mobility of the size markers. The polypeptide comprises the first 109 amino acids of fibronectin; it is not yet known if an additional methionine residue is present at the N-terminus of the final polypeptide. Throughout this specification this polypeptide is referred to as the r12 kD polypeptide or the r12 kD FBD.

B. Expression of a modified 12 kD (12 kD') partial FBD polypeptide

Plasmid pFN 975-25 (Figure 10), which expresses the full-length r31 kD FBD, was used to construct plasmid pFN 197-10 which expresses a modified r12 kD polypeptide (r12 kD') as shown in Figure 37. The fibronectin FBD sequence was modified to produce an NdeI site immediately after nucleotide 340. This plasmid was transformed into Escherichia coli strain A1645 and thence into Escherichia coli strain A4255. These transformed cells were found to be good expressors of the modified r12 kD partial FBD in amounts comprising about 5% of the total cellular protein. The polypeptide has a similar mobility to the unmodified 12 kD FBD as determined on reduced SDS polyacrylamide gels. The polypeptide comprises the first 109 amino acids of fibronectin followed by additional amino acids histidine and methionine; it is not yet known if an additional methionine residue is present at the N-terminus of the final polypeptide. This polypeptide is designated the r12 kD' polypeptide or the r12 kD' FBD.

C. Expression of a modified r12 kD FBD fused to the 33 kD cell binding domain

Plasmid pFN 197-10 which contains an NdeI site at the 3' terminus of the modified 12 kD FBD was used to construct a plasmid, designated pFN 202-5, which encodes the modified 12 kD FBD fused to the 33 kD cell binding domain (CBD). This construction was performed as shown in Figure 38 where the 33 kD CBD fragment was taken from plasmid pFN 137-2 (deposited in the ATCC under ATCC Accession No. 67910). Plasmid pFN 202-5 was transformed to Escherichia coli strain A1645 and thence to Escherich-

ia coli strain A4255 and is a good xpressor (8% of total protein). The 45 kD ppolypeptide consists of the 12 kD FBD fused to the 33 kD CBD (first 109 amino acids of FBD followed by amino acid residues histidine and methionine followed by the CBD commencing with serine; it is not yet known if an additional methionine residue is present at the N-terminus of the final polypeptide).

D. Expression of a 31 kD FBD polypeptide fused to the amino acid sequence DGRGDS

In order to obtain expression of a 31 kD FBD polypeptide fused at the carboxy terminus to the sequence asp-gly-arg-gly-asp-ser (DGRGDS) the following construction was made. Plasmid pFN 975-25 which expresses the 31 kD FBD was digested with PvuII and HindIII and ligated to a synthetic linker as shown in Figure 39. The resulting plasmid, designated pFN 195-4, was used to transform Escherichia coli strain A1645 and thence Escherichia coli strain A4255. These cells were found to be good expressors of the 31 kD-GRGDS polypeptide, at levels of about 8% of total cellular protein. The sequence of this polypeptide is described in the description of Figure 39.

E. Expression of a fused 31 kD FBD-33 kD CBD

In order to obtain expression of a "full length" r31 kD FBD polypeptide fused to the r33 kD CBD the following construction was made.

Plasmid pFN 975-25 which expresses the 31 kD FBD was digested with PvuII and HindIII, and the large fragment resulting was ligated to a synthetic linker and to the r33 kD cell binding domain obtained from plasmid pFN 137-2 aft r NdeI and HindIII digesti n (as shown in Figur

40). Th resulting plasmid, designat d pFN 194-2, encodes the r31 kD FBD linked to the 33 kD CBD. Plasmid pFN 194-2 was transformed to Escherichia coli strain A1645 and then to Escherichia coli strain A4255, and the resulting cells were low expressors of a 64 kD polypeptide which comprises the 31 kD FBD fused to the 33 kD CBD. The sequence of this polypeptide is described in the description of Figure 40.

Fermentation and growth conditions

The clone expressing the r12 kD FBD polypeptide was fermented in rich medium (yeast extract and casein hydrolysate) containing ampicillin. Growth was carried out at 30°C. Expression was obtained upon induction at 42°C for 2 hours and subsequently bacterial cell cake containing the r12 kD FBD polypeptide was obtained. Similarly, cell cake containing other proteins described above was obtained.

EXAMPLE 10

Refolding and Purification of Recombinant 20 kD and 12 kD Fibrin-Binding Polypeptides of Fibronectin.

The process for refolding and purification of the r20 kD and r12 kD polypeptides is made up of three stages:

1. Crude processing of the bacterial cake.

2. Refolding/reoxidation.

3. Purification.

1. Crude processing

1.1 Washing and extraction of the pellet: The bacterial cell cake, obtained as described in Example 2 for the r20 kD polypeptide and as described in Example 9 for the r12 kD polypeptide, is disrupted and washed essentially as for the r31 kD polypeptide (Example 5); however, changes were introduced in the extraction procedure used for both the r20 kD and the r12 kD polypeptides. The following is an example of the washing and extraction procedure performed on the bacterial cell cake of the r20 kD polypeptide; the r12 kD polypeptide is extracted in a similar way.

1.2 Procedure: Bacterial cake containing the r20 kD polypeptide was produced as described in Example 2 by fermentation of Escherichia coli strain A4255 harboring plasmid pFN 949-2. A portion of this bacterial cak (14.8 g) was suspended in 10 volumes of 50 mM Tris HCl, 50 mM EDTA (Buffer B), pH 7.5.

The suspension was homogenized for 15-30 sec nds at a medium speed, sonicated 3 times for 4 minutes with pulsing, and centrifuged at 15,000 rpm for 30 minutes. The pellet was resuspended in 2.4 volumes (36 ml) of Buffer B. Lysozyme (0.1 mg/ml) was added and the suspension was incubated in a water bath at 37°C for 2 hours with stirring. Triton X-100 was added to a final concentration of 1%, stirred at room temperature for 30 minutes and centrifuged. The pellet was resuspended three times in 148 ml of water (i.e., 10 times the volume of the original pellet), homogenized, stirred for 30 minutes at room temperature and centrifuged. The final pellet weighed approximately 1.5 g, i.e., only 10% of the original weight; however, both the r20 kD and the r12 kD polypeptides stay in the pellet, as evidenced by SDS-polyacrylamide gel-electrophoresis. The washed and extracted pellet was kept frozen at -20°C until further processed.

2. <u>Solubilization and refolding of the extracted pellet</u>

2.1 The reagents and procedure used for the refolding/-reoxidation are different in this case from those used for the r31 kD polypeptide. The extracted pellet of the r20 kD or the r12 kD polypeptide is dissolved in 6 M guanidine-HCl (GuCl) in the presence of 50 mM β-mercaptoethanol and, following a tenfold dilution, is allowed to reoxidize by air.

2.2 <u>Procedure</u>: The frozen r20 kD pellet (1.5 g) was solubilized and homogenized in 10 volumes of 10 mM Tris HCl, 5 mM EDTA (Buffer C), pH 8.0, containing additionally 6 M Guanidine-HCl. The sample was

reduced by th addition of 57 µl of undilut d β-mercaptoethanol (final concentration: 50 mM) and stirred in the absence of air, i.e., in a sealed container, for 30 minutes. It was then dripped at the rate of about 5 ml/min into 10 volumes (148 ml) of Buffer C, pH 8.0 and allowed to oxidize while being constantly and gently stirred, in an open beaker for 48-72 hours at room temperature. Although at this stage some polypeptide precipitation had already occurred, the suspension, including the precipitate, was dialyzed over 24 hours against 15 volumes of Buffer C, pH 8.5 with three changes of buffer. The dialysate was then subjected to centrifugation for 45 minutes at 15,000 rpm (22,500 x g) in a high-speed Beckman centrifuge equipped with a JA-17 rotor. This removes many contaminant proteins and aggregates of the r20 kD or r12 kD, which have been produced during reoxidation.

3. Purification and Characterization

Since the location of the heparin binding site within the fibrin binding domain was not known, it therefore could not be known in advance if the new shorter r20 kD and r12 kD polypeptides would bind to Heparin-Sepharose. However, we found that the shorter molecules did in fact bind to Heparin-Sepharose.

We found that there was no need for a phenyl-Sepharose column, as in the case of the r31 kD, in order to purify the reoxidized r20 kD or r12 kD polypeptides. In fact, the material could be dir ctly purified on Heparin-S pharose, but considerable improvement, with r spect to removal

of contaminants, incorrectly folded molecules and dimers, was achieved when the sample was chromatographed on a Q-Sepharose column before chromatography on a Heparin-Sepharose column. In some cases, the polypeptide was concentrated on a Pellicon system (Millipore Corp.), using membranes with appropriate cut-off points, i.e., 10 kD for the r20 kD polypeptide and 3 kD for the r12 kD polypeptide, prior to being loaded on the Q-Sepharose column. The Heparin-Sepharose column is also used for concentration of both polypeptides. The following is an example of the purification procedure used in the case of the r20 kD polypeptide.

3.1. Q-Sepharose Chromatography: One-third of the reoxidized r20 kD (47 ml) was applied to a 10 ml column of Q-Sepharose Fast Flow column, which had been pre-equilibrated in Buffer C, pH 8.5 at 1.2 ml/min flow-rate. The flow-through fraction was collected and saved (70 ml). The polypeptides which adhered to the column were eluted with Buffer C, pH 8.5 containing 0.5 M NaCl and the column was regenerated with 0.5 M NaOH.

3.2 Heparin-Sepharose Chromatography: The flow-through from the Q-Sepharose column was applied to a 10 ml column of Heparin-Sepharose pre-equilibrated in pH 8.5 buffer at a flow rate of 0.5 ml/min. The flow-through fraction contained mostly contaminants and incorrectly folded r20 kD polypeptide. The purified (>95% pure) r20 kD polypeptide was eluted in Buffer C, pH 8.5 containing 0.5 M NaCl and the c lumn was regenerated in th same buffer containing additionally 6M Guanidine-HCl.

Representative purification tables for th r20 kD (Table C) and r12 kD (Table D) polypeptides are provided.

3.3 Characterization: Supernatants from the processing of the bacterial cake for both the r20 kD and the r12 kD polypeptide, as well as aliquots from subsequent column fractions, were assayed for polypeptide and analyzed by SDS-polyacrylamide gel electrophoresis; their elution profiles were obtained on a Superose 12 column attached to either a FPLC or a HPLC. These profiles at various stages of the refolding, as well as of the purification, are shown for the r20 kD (Figure 52) and the r12 kD (Figure 53). The purified r20 kD or r12 kD polypeptides elute as single sharp bands. These profiles corroborate the results seen on SDS-PAGE gels under non-reducing conditions; the bands of both the 20 kD and the r12 kD polypeptides samples are non-diffuse, indicating a single molecular form. In the case of the r20 kD, the band of the non-reduced polypeptide runs (as in the case of the r31 kD polypeptide) faster than that of the reduced form; this is a similar effect to that seen in the case of the r31 kD polypeptide. However, no such difference is observed in the case of the r12 kD polypeptide.

These FBD polypeptides are available for radiolabeling in order to use them as radiopharmaceuticals for imaging of thrombi and atherosclerotic lesions. The FBD polypeptides may also be used for therapeutical inhibition or prevention of bacterial colonization and sepsis.

The advantages of using th smaller FBD polypeptides (r20 kD and r12 kD) for the above-mentioned purposes as opposed to using the larger r31 kD polypeptide is that we have developed after considerable effort a simpler method for the preparation of the smaller molecules, i.e., the methods described above for the refolding and purification of the r20 kD and r12 kD polypeptides are faster and easier than the method for refolding and purification of the r31 kD polypeptide. In addition, these methods result in a higher yield and a higher concentration of polypeptide than does the method for the r31 kD polypeptide.

TABLE C: PURIFICATION OF THE r20 kD PBD

Step	Volume (ml)	Protein Conc (mg/ml)	Total Protein (mg)	Purity[a] (%)	Amount of PBD (mg)	Yield (%)	Degree of purification
Solubilized & Reduced Pellet	14.8	12.4	183.5	35	64.2	100	1
Oxidized Supernatant	148	0.72	106.5	45	48.0	74.7	1.3
Oxidized Supernatant (1/3)	47		33.8		15.2		
Q-Sepharose Flow-through	70	0.20	14.0	80	11.2	54.9	2.3
Heparin-Sepharose 0.5 M NaCl	9	0.71	6.1	98	6.0	29.4	2.8

[a] Estimated from either SDS-PAGE gels under reducing conditions or from Sepharose 12 elution profiles.

This is a representative purification table for the refolding and purification of the r20 kD polypeptide, processed as described in Example 10, Sections 2 and 3.

TABLE D: PURIFICATION OF THE r12 kD PBD

Step	Volume (ml)	Protein Conc (mg/ml)	Total Protein (mg)	Purity[a] (%)	Amount of PBD (mg)	Yield (%)	Degree of purification
Solubilized & Reduced Pellet	100	6.66	666	10	66.6	100	1
Oxidized[b] Supernatant	500	0.20	100	58	58.0	87.1	5.8
Q-Sepharose Flow-through	500	0.13	65	80	52	78.1	8.0
Heparin-Sepharose 0.5 M NaCl	14	0.75	10.5	98	10.3	15.4	9.8

[a] Estimated from either SDS-PAGE gels under reducing conditions or from Sepharose 12 elution profiles.

[b] Concentrated on a Pellicon system.

This is a representative purification table for the refolding and purification of the r12 kD polypeptide, processed as described in Example 10, Sections 2 and 3.

EXAMPLE 11

Biological Activity of the r31 kD, r20 kD and r12 kD Fibrin Binding Domain Polypeptides

The biological activity of the r31 kD FBD was described (in Example 7) relating to its binding to fibrin clot *in vivo* and *in vitro*, binding to bacteria and binding to extracellular matrix. In this example, additional results relating to the r31 kD polypeptide are presented and the biological activity of the 20 kD and 12 kD FBD polypeptides is demonstrated.

In this Example the binding of the recombinant fibrin binding domains to fibrin clots was measured as follows:

Binding of ^{125}I-rFBD (r31 kD, r20 kD or r12 kD) to a preformed fibrin clot (two-step Reaction II)

Step 1 Formation of fibrin clot: This may be done in one of two ways:

Either (a) Incubation at 37°C of 20 µl citrated human whole blood with 5 mM $CaCl_2$, 1 unit/ml thrombin and PBS in a final volume of 250 µl. The reaction is terminated after 45 minutes by centrifugation and washing of the pellet (twice) with 1 ml PBS;

or (b) Incubation at 37°C of 20 µl non-citrated whole human blood ("naive" blood). The reaction is terminated after 150 minutes by centrifugation and washing as in (a).

Step 2 Binding of the ^{125}I-FBD polypeptide to the preformed fibrin clot

Clots are incubated at 37°C in a final volume of 250 µl PBS with ^{125}I-rFBD polypeptide. Other constituents may be added as indicated for each experiment. The binding reaction is terminated after 45 minutes by centrifugation and washing three times with PBS. The tubes containing the ^{125}I-rFBD - fibrin pellet were measured for radioactivity in a gamma counter.

Results

A. Metabolic stability of ^{125}I-labeled r31 kD FBD in rats: ex-vivo binding to fibrin versus TCA insolubility

As described in the description of Figure 44, rats were injected intravenously with r31 kD FBD labeled with ^{125}I and blood samples taken at intervals were added to Na citrate. Aliquots of the blood were treated as follows: either (a) treated with 20% TCA and the TCA insoluble counts were measured; or (b) incubated with preformed clot (using 20 µl whole blood from control rat); binding of the ^{125}I-31 kD FBD to preformed clot was measured under the conditions of the two-step Reaction II described above.

The radioactivity was measured by a gamma counter and the activity of each sample was calculated as a percentage of total cpm present in the reaction mixture.

The results demonstrat d in Figure 44 indicat a good correlation between the physical decay of the r31 kD (as measured by the decrease in TCA insolubility) and the functional decay (as measured by the decrease in ex-vivo binding of the r31 kD to a preformed fibrin clot.) However, at the initial stage of the comparative studies there are marked differences; at 30 min. the functional decay is several fold higher than the physical decay. These results, which suggest a much faster decrease of functional stability than physical degradation, can be explained since the main site for the covalent reaction of the FBD with the fibrin clot is the glutamine residue located at the extreme amino terminus of the FBD molecule at amino acid no. 3; this glutamine residue, being located in a 20 amino acid stretch outside the type 1 finger structure, is not protected from degradation by the tertiary structure, which is typical of the rest of the FBD domain.

B. Specificity of binding of r31 kD to fibrin: effect of transglutaminase

The covalent binding of the fibrin binding domain of plasmatic fibronectin to fibrin in a clot is mainly due to the reaction of amino acid no. 3 of fibronectin (glutamine) with fibrin; this binding reaction is enzymatically controlled by the enzyme transglutaminase which specifically recognizes the amino acid sequence containing this glutamine residue.

The following experiment was performed to investigate if transglutaminase is involved in the binding of the recombinant r31 kD FBD to clots. All the exogenous transglutaminase used in the experiments described in

this application is guin a-pig liver transglutaminase (Sigma).

The binding of 0.3 μM solution of the following molecules to preformed fibrin clot derived from 20 μl of whole human blood was measured in the presence and absence of transglutaminase using the two-step Reaction II described above: ^{14}C-putrescine-r31 kD FBD, ^{125}I-r31 kD FBD and ^{125}I-recombinant bovine growth hormone (control). The ^{14}C-putrescine-r31 kD protein complex where the glutamine residue at position 3 is blocked by covalent reaction with ^{14}C-putrescine, was prepared as follows:

A solution containing 3 μM r31 kD FBD, 10 mM $CaCl_2$, 0.015 units/ml transglutaminase and 60 μM ^{14}C putrescine (specific activity 100 mc/mmole) was incubated at 37°C for 5 hours. The amount of ^{14}C-putrescine incorporated into the 31 kD FBD was measured by TCA precipitation of an aliquot of this reaction solution and demonstrated the incorporation of an equivalent amount to 2.8-3 μM solution of ^{14}C-putrescine into the r31 kD protein; this indicates that more than 90% of the glutamine at position number 3 of the FBD covalently reacted with the ^{14}C-putrescine. The ^{14}C-putrescine r31 kD material was stored at 0°C and used within a few days without further treatment. The r31 kD FBD (prepared as described in Example 5) and the control recombinant bovine growth hormone analog (bGH) prepared as described in EPO Publication No. 131843 were labeled with ^{125}I using the ICl method described in Example 6.

Results

The c unts bound in the two-step R action II in the pres nce and absence f transglutaminase w re obtained

and th ratio of counts bound in the presence and absence of transglutaminase was calculated for each polypeptide tested (see Figure 45). This ratio differs dramatically when intact 31 kD FBD is compared to putrescine-FBD ("blocked" FBD) or to the control bGH. In the two latter cases the ratio of counts is close to 1 which shows that transglutaminase does not affect the binding and total cpm present in the clot is 10-15% of total cpm in the reaction). In the case of the intact 31 kD FBD the ratio is dramatically higher than 1 (in different experiments the ratio varied between 1.8-7 depending on the quality and freshness of the blood and the transglutaminase) and total cpm present in the clot is 40-70% of total cpm in the reaction) i.e., transglutaminase greatly increased the binding of r31 kD polypeptide to the clot.

These results indicate the strong effectiveness of unblocked glutamine at position number 3 for the binding of the r31 FBD polypeptide to the fibrin clot in the presence of transglutaminase.

Other experiments have shown that the addition of transglutaminase to the two-step Reaction II increases the binding of the r20 kD and r12 kD polypeptides to the clot, comparable to the effect observed with the r31 kD.

C. Characterization of r31 kD FBD-fibrin complex by SDS polyacrylamide gel electrophoresis

In order to determine the size of complex formed by the binding of r31 kD to a fibrin clot the following series of experiments (as described in the description of Figure 46) was undertaken. Clots were derived from either 20 µl whole human blood (A) or 250 µl of a solution of 0.8 µM human fibrinogen (B). In some of the fibrinog n experi-

ments dental coils (as described in Exampl 12) were added to the tubes together with the fibrinogen.

The binding of ^{125}I-r31 FBD to the fibrin clot was measured using the two-step Reaction II described above, in the presence of 0.15 μM ^{125}-r31 kD FBD and 5 mM $CaCl_2$. The reaction was terminated by three times washing with PBS. The pellet, after the various treatments described below, was centrifuged and 15 μl aliquots of the supernatant (i.e., the soluble material) were electrophoresed on polyacrylamide gels which separates the material of molecular weight >10^6 from molecular weight >10^5 and from lower molecular weight materials. An autoradiogram was produced (Figure 46) which shows the following: in the presence of transglutaminase high molecular weight forms of r31 kD - fibrin complex appears which are resistant to boiling in ths presence of the strong ionic detergent SDS and ß-mercaptoethanol, which reduces S-S bonds.

Additionally, Figure 46 demonstrates that when 4M urea is included in the boiling reaction the very high molecular weight forms (>10^6) are quantitatively converted to the intermediate molecular weight forms (>100,000) as expected for hydrophobic bonded aggregates of high molecular weight fibrin clots. The amount of free r31 kD polypeptide in the clots is normally small; this is the material released on boiling with phosphate-saline buffer only. The resistance of the intermediate molecular weight forms to additional treatment with urea supports the involvement of a covalent linkage between the ^{125}I-r31 kD and the fibrin.

D. Effect of fibronectin and heparin on the binding of ^{125}I-r31 kD to preformed fibrin clots

(i) Effect of fibronectin (FN)

Human plasma contains substantial levels of FN (300 μg/ml) which potentially could compete with the binding of ^{125}I-r31 kD polypeptide to preformed clots. Such competition may affect the efficiency of clot radiolabeling and subsequently the imaging process. To examine the effect of FN, ^{125}I-r31 kD (0.15 μM) was added together with purified FN (1 μM) to a preformed clot in PBS (Figure 47A). Although FN was added in a molar excess of 7 relative to ^{125}I-r31 kD, the binding of the latter polypeptide was only slightly affected (20% inhibition). The observation that excess FN does not compete with ^{125}I-r31 kD binding could be interpreted in two ways: the number of sites for crosslinking onto the clot is in excess to accommodate both FN and ^{125}I-FBD, or the affinity of FBD to the clot is much higher than that of FN. Based on several observations, we believe that both excess binding sites and higher affinity of the ^{125}I-r31 kD enable its binding to the clot in the presence of plasma concentrations of FN.

(ii) Effect of heparin

Some radioscintigraphic agents such as ^{111}In-labeled platelets and labeled fibrinogen are ineffective in the presence of therapeutic heparin. It was important, therefore, to analyze the effect of heparin on the incorporation of ^{125}I-r31 kD to the clots. The results shown in Figure 47B indicate that heparin has no significant effect on the binding of r31 kD FBD to preformed

clots. Other experiments showed that the sam amount of heparin affects dramatically the binding of r31 kD to a fibrin clot during its formation, i.e., Reaction I (see Example 7).

E. Comparison of the binding of various recombinant FBD polypeptides and plasmatic FBD to preformed clots

To compare the binding to preformed clots of the various recombinant FBD polypeptides (r31 kD, r20 kD and r12 kD) and plasmatic 31 kD FBD, a series of experiments using the two-step Reaction II was carried out as described in Figure 48. The results show that the plasmatic 31 kD binds to a similar level as the r31 kD whereas the r20 kD and r12 kD polypeptides both bind at about half the level of the larger (31 kD) molecule. The level of binding of the r20 kD and r12 kD polypeptides is still sufficiently high to demonstrate the potential of radiolabeled r20 kD and r12 kD polypeptides as radiopharmaceuticals for thrombus imaging. Similar experiments using the r31 kD-DGRGDS polypeptide (Example 9, D and Figure 39) showed that it binds at about the same level as the r31 kD.

F. Binding of ^{125}I-r12 kD to fresh or frozen clots

In order to study the effect of freezing the clots prior to use in binding experiments with FBD polypeptides, the following experiment was carried out. Fibrin clots were either used fresh or after storage at -70°C in a two-step Reaction II binding experiment with ^{125}I-r12 FBD, prepared as described in Example 10.

The exp riment was carried out as d scribed in the Description of Figure 41 in the pres nce or absence of

transglutaminase. Figure 49 shows that there is little significant effect of freezing on the abilities of clots to bind r12 kD FBD. Normally, frozen clots without added transglutaminase yield binding results similar to fresh clots in the presence of transglutaminase; there is no effect on the binding reaction when exogenous transglutaminase is added to frozen clots, probably because of the release of endogenous transglutaminase from the frozen red blood cells.

As noted in Section B above, there is a wide range of response to addition of exogenous transglutaminase in Reaction II.

G. Conditions for binding of ^{125}I-r31 kD FBD to preformed clots

To investigate the conditions for binding of ^{125}I-r31 kD to preformed clots, the following series of experiments were carried out. The binding of ^{125}I-r31 kD polypeptide to preformed clots formed from citrated or "naive" blood was examined, using the two step Reaction II method, and in the presence or absence of various constituents (calcium, hirudin, transglutaminase). The results are shown in Figure 50. The pattern of results using the "citrated" blood or "naive" blood clots is similar although the binding of the r31 kD polypeptide is higher to citrated blood.

Hirudin (Sigma) is a specific inhibitor of any thrombin-mediated reaction and the hirudin was therefore added in order to investigate the effect of thrombin on the binding reaction (step 2). No effect of hirudin was shown and therefore thrombin has no effect on the binding of the r31 kD polypeptide to the clot. However, the sam

amount of hirudin totally inhibits th binding when added at step 1 where fibrin is formed from fibrinogen, as was expected.

These results also show that exogenous transglutaminase increases the binding of r31 kD FBD to clots and furthermore that this transglutaminase reaction is dependent on the presence of calcium ions. Since the exogenous transglutaminase used is tissue transglutaminase (in its active form) we expect that the serum transglutaminase, factor XIII, which has to undergo activation by thrombin to form factor XIIIa, will be highly sensitive to hirudin inhibition.

H. <u>Conditions for binding ^{125}I-r31 kD FBD to the extracellular matrix (ECM)</u>

The binding of ^{125}I-r31 kD to the extracellular cell matrix of endothelial cells (ECM) was demonstrated in Example 7 (Section 9) and in Figure 24. The binding was now further characterized by examination of the binding of 0.3 μM ^{125}I-r31 kD FBD to ECM in the presence and absence of exogenous transglutaminase; additionally the binding in the presence of transglutaminase was examined in the presence of each of heparin, fibronectin or spermidine.

The results of these experiments are shown in Figure 51 which demonstrates that the binding of the r31 kD FBD to ECM is increased by the addition of transglutaminase. Heparin has no significant effect on the binding whereas spermidine, a known inhibitor of transglutaminase, inhibits the binding. Collagen also inhibits the binding, suggesting th possibl involv ment of collagen as an acceptor mol cule on th matrix of th endothelial

c lls. Fibronectin has littl ffect on the binding reaction.

These results give more support to the potential use of radiolabeled recombinant FBD polypeptides as radiopharmaceuticals for imaging the initial plaque formation in denudated blood vessels.

EXAMPLE 12

Uptake of Recombinant ^{125}I-31 kD FBD and Fragments Thereof by Stainless Steel Coil-Induced Venous Thrombi in Rats

The stainless steel coil-induced venous thrombus model in rats was used to study the uptake of labeled r31 kD, r20 kD and r12 kD FBD polypeptides. The model employed was as described by Maffrand et al. [Thrombosis and Haemostasis 59: 225-230 (1988)].

Experimental Details

A. Investigation of the uptake of ^{125}I-31 kD by the stainless steel coil-induced venous thrombus

Wistar-derived female rats (200-250 g) were anaestetized by Ketamine HCl plus Xylazin HCl. A midline abdominal incision was made and the inferior vena cava was exposed. A stainless steel wire coil (a dental paste carrier, fine No. 31, 21 mm long) was inserted into the lumen of the vein at the site just below the junction, and the incision was sutured. Each inserted device was individually weighed before insertion and each weight recorded. Three hours after the operation, the animals were given an i.v. injection of 1 ml of 0.9% NaI solution in order to saturate the thyroid iodide pool. One hour later, the rats received an i.v. injection of ^{125}I-r31 kD FBD (5 x 10^6 cpm; 100 µg/kg). The r31 kD polypeptide was labeled as described in Example 6. At 24 hours after the administration of the labeled polypeptide, blood was drawn by cardiac puncture, and the rats were sacrificed. The segment of the vein carrying the coil was removed while taking car t drain away all residual blood. In one group, the segments carrying th c il were weighed as

such and taken for measurement of radioactivity (the "Thrombus in Situ" group). In another group the vein sections were incised longitudinally, and the coils carrying the thrombi were carefully removed, weighed and the radioactivity was measured. The blood radioactivity levels were measured using peripheral blood.

Calculation of the results:

In the two groups, the initial weight of each coil was subtracted from its final weight, and the specific radioactivity in each case was calculated by division of the cpm value by the net weight. The specific activity of the peripheral blood samples was also calculated.

Results:

At 24 hours, the blood levels of radioactivity were around 5000-10,000 cpm/g, while in the isolated blood clot the specific radioactivity was around 300,000 cpm/g, i.e., 30 to 60 fold higher (see Figure 42). When the entire segment of the vein carrying the clot was included in the analysis, and a so-called "specific radioactivity" value calculated, the resultant values were 4-5 fold higher than those of the blood, thus indicating that a good signal-to-noise ratio may be obtained for gamma-camera imaging of blood clots in vivo using labeled r31 kD FBD.

The effect of heparin pretreatment was studied in this model. This kind of experiment is essential because patients that are candidates for thrombus imaging are usually treated with this anticoagulation agent. In order to study this qu stion, a gr up of rats w re treat d with heparin (500 units/rat intravenously) 10

minutes before administration of th labeled polypeptide. This treatment of heparin did not affect the uptake of label, as measured 24 hours later.

These results demonstrate that thrombus imaging using the FBD of FN may be done in the presence of heparin.

B. Comparison of recombinant 12 kD, 20 kD and 31 kD-FBD polypeptides in the stainless steel coil-induced venous thrombus model

The three recombinant polypeptides were labeled with ^{125}I as described in Example 6 and utilized in the rat model as described in A above. The results, shown in Figure 43, indicate that each of the three molecules was specifically localized in the clots as compared to the blood, by comparing the specific radioactivities; the specific radioactivity of the clots appeared to be higher with the longer molecules than the shorter polypeptides (143,000, 78,500 and 63,000 cpm/g clot for the r31 kD, r20 kD and r12 kD polypeptides, respectively), but the differences were not statistically significant. The specific radioactivity values for blood (after 24 hours) were similarly related to the molecular size (7040, 5016 and 3300 cpm/g for the r31 kD, r20 kD and r12 kD polypeptides, respectively) and might reflect differences in the blood clearance rates of these molecular species. Hence, the calculations of the ratio of clot to blood specific radioactivity resulted in values that were similar for the three different polypeptides, and ranged around 20. These results suggest that all three FBD species (or other fragments of the FBD) could serve for thrombus imaging.

EXAMPLE 13

Labeling of the fibrin binding domain polypeptides for imaging atherosclerotic lesions and thrombi

The fibrin binding domain polypeptides described in this application (the r31 kD, the r20 kD and the r12 kD polypeptides), or other FBD fragments, may be radioactively labeled to carry a radiotracer to a thrombus in order to permit its external detection by gamma camera imaging. This application discloses in Example 6 the labeling of these three polypeptides by means of iodine-125 (^{125}I), which has a long half life of 60 days.

Another radioiodine is iodine-131 (^{131}I) which may be used to label the FBD polypeptides using known methods such as described by Uehara et al (1). However, ^{131}I has a relatively long half life of 8 days.

Optimally, a radiopharmaceutical for clinical imaging of atherosclerotic lesions and thrombi should yield positive results within the first few hours after injection (33). For such a test a shorter lived radiolabel could be used. Recent studies have suggested that indium-111 (^{111}In) or technetium-99m (^{99m}Tc) may be more suitable as radiotracers, since they have half-life of 67 hours and 6 hours, respectively (32); another short-lived low energy label is iodine-123 (^{123}I) with a half-life of 13.3 hours.

We have therefore labeled the r31 kD, r20 kD and r12 kD polypeptides and the plasmatic 31 kD fragment by means of ^{111}In using the method described for human serum albumin by Hnatowich, D.J., Layne, W.W. and Childs, R.L. in J. Appl. Radiat. Inst. 33: 327 (1982). Pr liminary experi-

ments have shown that the labeled FBD polypeptides bind to preformed thrombi in vitro, measured by the two-step Reaction II (Example 11) and to thrombi in vivo measured by the model described in Example 12, and giving a high thrombus:blood ratio in the range of 80-200 after 24 hours.

The labeling of the FBD polypeptides by ^{99m}Tc may be carried out using known methods (21, 33, 34, 35). ^{99m}Tc is a very suitable diagnostic single photon radionuclide because of its short half-life, a detection level of 140 KeV with the gamma counter, no particulate radiation and inexpensive, convenient availability. These attributes allow the routine administration of doses of 30m Ci that result in high photon-flux levels facilitating lesion detection by single photon emission computerized tomography (32, 35).

Other radiolabels which may be used to label the FBD polypeptides include krypton-81m (^{81m}Kr) and xenon-133 (^{133}Xe), which has a half-life of 5.3 days, as reviewed by Knight (4). Another potential radiolabel is gallium-67 (^{67}Ga) as described by Yamamoto (36); ^{67}Ga has a half-life of 78 hours.

NMRI, ultrasound and X-ray imaging with metal chelates are described in U.S. Patent 4,647,447. In addition, antibody coupling with metal chelates is mentioned at column 7, line 42. Monoclonal antibodies labeled with polymeric paramagnetic chelates and their use in NMRI methods have also been described [Shreve, P. et al., Magnetic Resonance in Medicine 3: 336-340 (1986) and Brady, T. et al. in Proceedings of the Society of Magnetic Resonanc in Medicine, Second Annual Meeting, Soc. of Magnetic Resonance in Medicin , Inc., San

Francisco, p 10, 1983 refer nced by Koutcher, J. et al., J. Nucl. Med. 25: 506-513 (1984)].

REFERENCES

1. Uehara, A., et al., J. Nuclear Med. 29: 1264-1267 (1988).

2. Zoghbi, S.S., et al., Invest. Radio. 20: 198-202 (1985).

3. Kakkar, V.V., et al., Lancet 1: 540-542 (1970).

4. Knight, L.C., Nuclear Med. Commun. 9: 849-857 (1988).

5. Knight, L.C., Nuclear Med. Commun. 9: 823-829 (1988).

6. Som. P., et al., J. Nuc. Med. 27: 1315-1320 (1986).

7. Palabrica, T.M., et al., Proc. Nat. Acad. Sci. USA 86: 1036-40 (1989).

8. Akiyama, S.K. and Yamada, K.M., Adv. Enzymol. 57: 1-57 (1987).

9. Pierschbacher, M.D., et al., J. Biol. Chem. 257: 9593-9597 (1982).

10. Pande, H. and Shively, J.E., Arch. Biochem. Biophys. 213: 258-265 (1982).

11. Hayashi, M. and Yamada, K.M., J. Biol. Chem. 258: 3332-3340 (1983).

12. Sekiguchi, K. and Hakomori, S.-I., Proc. Natl. Acad. Sci. USA 77: 2661-2665 (1980).

13. Ruoslahti, E., et al., J. Biol. Chem. 256: 7277-7281 (1981).

14. Owens, R.J. and Baralle, F.E., EMBO J. 5: 2825-2830 (1988).

15. Obara, M., et al., FEBS Letters 213: 261-264 (-1987).

16. Obara, M., et al., Cell 53: 699 (1988).

17. Ichihara-Tanaka, K., et al., J. Biol. Chem. 265: 401-407 (1990).

18. Mandel, et al., Principal and Practice of Infectious Disease 2: 1531-1552 (1979).

19. Proctor, R.A., et al., J. Biol. Chem. 255: 1181-1188 (1980).

20. Eldor, A., et al., Thrombosis and Haemostatis 56(3): 333-339 (1986).

21. Fritzberg, A.R., Nucl. Med. 26: 7-12 (1987).

22. Young, R.A. and Davis, R.W., Proc. Natl. Acad. Sci. USA 80: 1194-1198 (1983).

23. Hugh, T., et al., In DNA Cloning: A Practical Approach (D. Glover, ed.), IRL Press, Oxford (1984).

24. Vog 1, et al., Proc. Natl. Acad. Sci. USA 69: 3180-3184 (1972).

25. Bagly, D., et al., Methods in Enzymol. 45: 669-678 (1976).

26. Wagner and Hynes, J. Biol. Chem. 254: 6746-6754 (1979).

27. McDonagh, R.P., et al., FEBS Lett. 127: 174-178 (1981).

28. Mosher, et al., J. Biol. Chem. 255: 1181-1188 (1980).

29. Russel, P.B., et al., J. Clin. Micro. 25: 1083-1087 (1987).

30. Bolton, A.E. and Hunter, W.M., Biochem. J. 133: 529 (1973).

31. Obara, et al., Cell 53: 649-657 (1988).

32. Fritzberg, A.R., et al., Proc. Natl. Acad. Sci. 85: 4025-4029 (1988).

33. Knight, L.C., et al., Radiology 173: 163-169 (-1989).

34. Wasser, M.N.J.M., et al., Blood 74: 708-714 (1989).

35. Burger, J.J., et al., Methods in Enzymology 112: 43-56 (1985).

36. Yamamoto, K., et al., Eur. J. Nucl. Med. 14: 60-64 (1988).